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Biological activity of well defined hydantoin derivatives on efflux pump systems of bacteria and cancer cells

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Biological activity of well defined hydantoin derivatives on efflux pump systems of bacteria and cancer cells

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Abbreviation list

ABC-transporter - ABC-binding cassette transporter superfamily

A. baumannii - *Acinetobacter baumannii*

ADME – Absorption, distribution, metabolization and excretion kinetics of the organism

B. subtilis - *Bacillus subtilis*

CCCP - Carbonyl cyanide *m*-chlorophenylhydrazone

CDC – Center for disease prevention and control

CFTR - Cystic fibrosis transmembrane regulator protein

CGS - Cellular glutathione system

DMSO –Dimethyl sulfoxide

DNA – Desoxyribonucleic acid

EB – Ethidium bromide

E. coli – Escherichia coli

ECDC – European Centre for Disease Prevention and Control

E. faecalis – Enterococcus faecalis

E. aerogenes – Enterobacter aerogenes

EPI – Efflux pump inhibitor

FACS - Fluorescence Activated Cell Sorting

FDA – U.S. Food and Drugs Administration

H. pylori – Helicobacter pylori

ICU – Intensive care unit

IM - Inner membrane

K. pneumoniae – Klebsiella pneumoniae

L. lactis – Lactococcus lactis

LPS - Lipopolysaccharide

MALT - mucosa-associated lymphoid tissue lymphoma

MATE - Multidrug and toxic compound extrusion superfamily

MDR – Multidrug resistance

MFP – Membrane fusion protein

MFS – Major facilitator superfamily

MIC – Minimum inhibitory concentration

MRSA - Methicillin-resistant *Staphylococcus aureus*

MTT - Thiazolyl blue tetrazolium bromide

M. tuberculosis – Mycobacterium tuberculosis

NBD – Nucleotide binding domain

OM – Outer membrane

OMF – Outer membrane factor

OMP - Outer membrane protein

PAR – Parental cells

P. aeruginosa – Pseudomonas aeruginosa

PDR – Pandrug-resistance

P-gp – P-glycoprotein

PMH – phenyl-methylene hydantoin

PPI - Proton pump inhibitor

PMF – Proton motive force

QSAR - Quantitative structure-activity relationship

RF – Relative fluorescence

RFF – Relative final fluorescence

RNA – Ribonucleic acid

RND – Resistance-nodulation division superfamily

S. aureus – Staphyloccocus aureus

SDS - Sodium dodecyl sulphate

S. Enteritidis – *Salmonella* Enteritidis

SMR – Small multidrug resistance superfamily

S. pneumoniae – Streptococcus pneumoniae

S. typhi – Salmonella typhi

TA - Teichoic acid

TM - Transmembrane

TMD – Transmembrane domain

TMH - Transmembrane helix

TMS – Transmembrane segments

VEGF - Vascular endothelial growth factor

V. cholera – Vibrio cholerae

XDR – Extensive drug-resistance

WHO – World Health Organization

Resumo

A multi-resistência a antibióticos e medicamentos usados em quimioterapia é um dos grandes problemas com os quais as instituições de saúde se debatem hoje em dia. A acção provocada por bombas de efluxo é uma das suas causas. Estas bombas têm uma importância fundamental, uma vez que, ao expelirem todo o tipo de tóxicos para o exterior das células, também expelem medicamentos, fazendo com que estes não tenham o efeito desejado dentro delas.

As bombas de efluxo são transportadores que se encontram nas membranas de todo o tipo de células. Existem dois grandes tipos de bombas de efluxo: as primárias e as secundárias. As primeiras conferem multi-resistência principalmente em células eucariotas, como as células do cancro em humanos, tendo como função a mediação da repulsa de substâncias tóxicas por intermédio da hidrólise de ATP. A primeira a ser descoberta e mais estudada destas bombas foi a ABCB1 que é o gene que codifica a glicoproteína-P (P de permeabilidade). Enquanto as secundárias, que são a maior fonte de multi-resistência em bactérias, promovem a extrusão de substâncias tóxicas através da força motriz de protões. Neste tipo de bombas são conhecidas quatro famílias principais, das quais uma das mais importantes é a superfamília RND, uma vez que inclui a bomba AcrAB-TolC, que é muito importante no metabolismo xenobiótico de bactérias Gram-negativas, nomeadamente a *E.coli*.

Com o objectivo de reverter a multi-resistência, tanto em células eucariotas como procariotas, têm-se desenvolvido estratégias de combate que envolvem a descoberta de substâncias que inibam as bombas de efluxo. Assim sendo, ao longo dos tempos têm sido descobertas variadas substâncias que cumprem este objectivo. É o caso, por exemplo, dos derivados de fluoroquinolonas usados como inibidores de bombas de efluxo em bactérias ou do Tamoxifen, utilizado na terapia de pacientes com cancro da mama.

Um dos grupos de substâncias estudados para o desenvolvimento de possíveis compostos que actuem como reversores de multi-resistência são os compostos derivados de hidantoínas. Estes, são conhecidos por possuírem uma grande variedade de propriedades bioquímicas e farmacológicas, sendo portanto usados para tratarem algumas doenças em humanos, como a epilepsia. Nestes, estão englobados compostos com actividade anti-convulsão que constitui a sua grande mais-valia e, dependente da substituição no anel que os constitui, uma grande variedade de outras propriedades farmacológicas como a anti-fungica, a anti-arritmica, a anti-viral, a anti-diabética ou por exemplo a antagonização de determinados receptores, como os da serotonina. Apesar de pouco usados em estudos experimentais para desenvolver substâncias anti-carcinogénicas, existem alguns estudos com este efeito.

Objectivos: O presente projecto envolve o estudo de bombas de efluxo primárias e secundárias, em células eucariotas e procariotas, respectivamente. Em bactérias, foram usados quatro modelos experimentais: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *E. coli* AG 100 e *Salmonella* Enteritidis NCTC 13349. Em células de cancro foram usadas, células T de linfoma de rato parentais e células T de linfoma de rato transfectadas com o gene humano MDR-1.

O principal objectivo deste estudo foi a pesquisa de novos moduladores de bombas de efluxo presentes em bactérias e células do cancro, tentando assim contribuir para o desenvolvimento de novos agentes farmacológicos que consigam reverter a multi-resistência a medicamentos. Assim sendo foram testados trinta compostos derivados de hidantoínas: **SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, P10, P11, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, KK-XV, Thioam-1, JHF-1, JHC-2, JHP-1, Fur-2, GL-1, GL-7, GL-14, GL-16**, **GL-18**.

Como forma de atingir estes objectivos, a actividade biológica dos trinta compostos derivados de hidantoínas foi avaliada nas quatro estirpes de bactérias da seguinte forma: foram determinadas as concentrações mínimas inibitórias dos trinta compostos como forma de definir as concentrações em

que os compostos seriam utilizados. Os compostos foram posteriormente testadas com um método fluorométrico de acumulação de brometo de etídeo, que é um substrato comum em bombas de efluxo bacterianas, desenvolvido por Viveiros *et al*.

A actividade biológica dos compostos derivados de hidantoínas nas células de cancro foi demonstrada por diferentes métodos. O efeito anti-proliferativo e citotóxico dos trinta compostos foi avaliado nas células T de linfoma de rato transfectadas com o gene humano MDR-1 pelo método de thiazolyl de tetrazólio (MTT). Como o brometo de etídeo também é expelido pelos transportadores ABC, estes compostos foram posteriormente testados com um método fluorométrico de acumulação de brometo de etídeo desenvolvido por Spengler *et al* nos dois diferentes tipos de células eucariotas.

Resultados: A maioria dos compostos derivados de hidantoínas foi eficaz na modulação de bombas de efluxo, nas duas estirpes de bactérias Gram-negativas e nos dois diferentes tipos de células T de linfoma. Em contraste com estes resultados, nas duas estirpes de células Gram-positivas, a maioria dos compostos tiveram pouco efeito na inibição de bombas de efluxo ou até nenhum, em muitos dos casos. De uma maneira geral os melhores compostos nas diferentes estirpes de bactérias foram: **Thioam-1**, **SZ-2**, **P3**, **Rw-15b**, **AD-26**, **AD-29**, **GL-18**, **GL-7**, **KF-2**, **SZ-7**, **MN-3**, **GL-16** e **GL-14**. Foram portanto estes os compostos que provocaram maior acumulação de brometo de etídeo, inibindo assim com maior eficácia as bombas de efluxo.

No presente estudo, a maioria dos compostos conseguiu inibir a resistência provocada pela bomba de efluxo ABCB1, tanto nas células parentais bem como nas células que sobre-expressam esta bomba, causando a acumulação de brometo de etídeo dentro das células. As células que sobre-expressam a bomba ABCB1 foram posteriormente testadas com citometria de fluxo que é a técnica padrão para pesquisa de inibidores de bombas de efluxo.

Os compostos que foram mais efectivos na inibição da bomba ABCB1, causando assim maior acumulação de brometo de etídeo nas células que sobre-expressam esta bomba foram: **PDPH-3**, **GL-7**, **KK-XV**, **AD-29**, **Thioam-1**, **SZ-7**, **KF-2**, **MN-3**, **RW-13**, **LL-9**, **P3**, **AD-26**, **JH-63** e **RW-15b.** Este facto não corroborou totalmente os resultados da citometria de fluxo uma vez que os moduladores que provocaram maior inibição da bomba ABCB1 foram o **MN-3**, **JH-63** e o **BS-1**, sendo que o último não foi seleccionado como um bom composto usando o método fluorométrico de acumulação de brometo de etídeo.

Conclusão: Os compostos derivados de hidantoínas testados tiveram maior efeito nas estirpes de bactérias Gram-negativas do que nas Gram-positivas. Relativamente às células eucariotas, as estruturas mais activas apresentam substituintes aromáticos bem como alguns fragmentos aminicos terciários.

I – INTRODUCTION

1. Historical background of the control of bacterial infections

In spite of the great efforts on health development, to combat infectious diseases is one of the great concerns of health care institutions. Taking in consideration the United Nations Millenium objectives, one of the most important needs for human populations is the fight against these microorganisms, reducing thus the deaths of thousands of lives (WHO). From their discovery, more or less sixty years ago, antibiotics played a huge role in the development of medicine, contributing astonishingly to the wellbeing and evolution of the society, becoming thus one of the pillars of modern medicine (MILLER *et al,* 2008).

In the year of 1546, Hieronymus Fracastorius published a book called "De cotagione", where he described three modes of disease spread: direct contact with infected persons, indirect contact with fomites and airborne transmission. In the XIX century, Ignaz Philipp Semmelweis, endeavoured the practise of hands washing before surgeries in order to reduce the spread of infection, which was the start of antiseptic prophylaxis. These measures were not well received at that time; however many deaths by hospital gangrene would have been avoided. It was in the end of the XIX century when infection control measures, such as hands washing or boiling of surgical instruments before use started to be common (nobelprize.org).

In 1864, Pasteur, with his work on fermentation, unveiled the role of microorganisms as agents of infectious disease. Shortly after, Joseph Lister was the first to denote the connection between Pasteur's work and the suppuration of wounds in surgical settings. In 1867 he published his work on antisepsis and then started to apply carbolic acid to open fractures. With this new measure, the healed suppurations plummeted, which dropped the deaths from amputation from 45 to 15 % (nobelprize.org).

Paul Ehrlich is one of the greatest contributors to the research on infectious diseases. In the beginning of the XX century he presented an idea in his doctoral thesis: “The chemical constitution of drugs used must be studied in relation to their mode of action and their affinity for the cells of the organisms against which they were directed”. In collaboration with Hata, he tested a drug on syphilis-infected rabbits that was very effective and known under the name Salvarsan (SYKES, 2010).

In 1929, Sir Alexander Fleming was searching for potential antibacterial compounds. He noted that a patch of the mould *Penicillium notatum* could grow on a *Staphylococcus* plate. He also denoted that around the mould, the *Staphylococcus* strain could not grow. However, just Ernst Chain and Howard Florey could make the mass production of penicillin possible. The substance, highly effective against some of the most prevalent infectious agents, was then used in World War II, saving thousands of lives. This discovery remains one of the most remarkable advents in medicine. In parallel, Gerhard Domagk discovered the first synthetic molecule, a sulphonamide derivative with antibacterial properties. He used this drug to cure his daughter’s streptococcal infection, revealing thus the powerful properties of the sulphonamide derivative, which came into clinical usage in the 30’s, under the name Prontosil. Both penicillin and prontosil discoveries paved the way to the advent of more natural and synthetic antibiotics (SYKES, 2010; TODAR, 2004).

Previously, in the XX century, an antibiotic was defined as a chemical substance that was produced by a microorganism and, in dilute solutions, could inhibit the growth of other microorganisms. That definition was then broaden in order to include similar inhibitory substances produced not solely by microorganisms. Today, the antibiotics combined with improvements in sanitation, housing and nutrition alongside the advent of widespread vaccination programs, have led to a dramatic drop in the once common infectious diseases that formerly killed lots of people. Since 1977, the WHO has been publishing the so called Model Lists of Essential Drugs, regularly updated, which have been of great help, decreasing the number of malpractices regarding antimicrobial therapy (WHO).

However, the world has been assisting to the re-emergence of infectious diseases, mainly because of the increasing antibiotic resistance. The key factors driving this threat are the following: migration of people, animals and goods, increased industrialization and increased antibiotic usage (HAWKEY & JONES, 2009). While in the most developed countries the problem is related to the overuse and abuse of antibiotics (agriculture, tetracyclines in animal husbandry), in poorer countries it is related to the lack of proper medication (WHO).

In order to overcome the emerging antibiotic resistance, the World Health Organization launched the WHO Global Strategy for Containment of Antimicrobial Resistance. This strategy is directed to reduce the spread of resistance in various ways. The interventions should be centered in groups of people whose behavior contribute to resistance, which have significant impact at both national and international levels. These interventions are thus directed to people and institutions as well, e.g. from the pharmaceutical industry to consumers and managers of hospitals. On the other hand, the introduction of legislation governing the development, licensing, distribution and sale of antimicrobials is also of great importance (WHO).

2. Antibiotics and their importance to human medicine

2.1 Importance of the discovery of antibiotics

Physicians around the globe have realized soon that bacteria might become resistant to antibiotics. Concomitant with the first description of the clinical use of penicillin there was a report of an enzyme (named penicillinase) that conferred resistance to penicillin (HAWKEY, 2008a). But at that time, a period of ever more number of successfully introduced drugs into clinical practice, it was not too much alarming. The 1940's and 1950's were the most prolific to the antibiotics discovery, with the introduction of several classes of drugs: aminoglycosides, β-lactams,

chloramphenicol, tetracycline, macrolides, glycopeptides, streptogramins and lincosamides. Rifamycin and nalidixic acid have been used since the 1960's. The arrival of new classes of therapeutically useful antibiotics lasted two more decades: oxazolidinones (linezolid) and lipopetides (daptomycin) (ECKER & CHIBA, 2010; REDDY *et al,* 2009).

The accelerated resistance of important human pathogens has been the main cause for the lack of antibiotics discovery. The increasing morbidity and mortality rates associated with bacterial infections are some of the consequences (HAWKEY & JONES, 2009; SAIER *et al*, 1998; NIKAIDO, 2001). Two of the strategies that have been used to overcome this situation is the improvement of old antibiotics and the design of new generations of antibiotics, with the help of new tools, e.g., bacterial genomics (NIKAIDO, 2001).

2.2 Mode of action of antibiotics

Most of the antimicrobials used for the treatment of bacterial infections can be categorized according to their principal mode of action: 1) interference with cell wall synthesis, 2) inhibition of protein synthesis, 3) interference with nucleic acid synthesis, 4) inhibition of a metabolic pathway and 5) disruption of the bacterial membrane structure (TENOVER, 2006). Table 1 presents some examples according to the antibiotics mode of action.

Table 1: Mode of action of antibiotics (TENOVER, 2006**)**

Mode of action	Examples
Interference with cell wall synthesis	**β-Lactams:** penicillins, cephalosporins, carbapenems, monobactams **Glycopeptides:** vancomycin, teicoplanin
Protein synthesis inhibition	macrolides[1], cloramphenicol[1], clindamycin[1], linezolid[1], aminoglycosides[2], tetracyclines[2], mupirocin[3]
Interference with nucleic acid synthesis	fluoroquinolones[4], rifampin[5]
Inhibition of a metabolic pathway	sulfonamides, folic acid analogues
Disruption of bacterial membrane structure	Polymyxins, daptomycin

[1]bind to 50S ribosomal subunit, [2]bind to 30S ribosomal subunit, [3]bind to bacterial isoleucyl-transfer RNA synthetase, [4]inhibit DNA synthesis, [5]inhibit RNA synthesis

2.3 Antibiotic resistance: emerging problems

WHO defines antimicrobial resistance as the use of an antimicrobial, in any dose and over any time period that causes a selective pressure on microbial populations. Antimicrobials become thus less effective against resistant microorganisms that can spread (WHO).

Selective pressure, which is the driving force of evolution and natural selection caused by the prolonged use of antibiotics, had big consequences on the increase of resistance (HAWKEY, 2008a; FALAGAS & BLIZIOTIS, 2007). This contributed to unveil strains of bacteria that are no longer susceptible to the conventional antimicrobial therapy. In particular, staphylococci, enterococci, *Klebsiella pneumoniae* and *Pseudomonas ssp*, that are becoming commonplace in healthcare institutions.

Concerning the so called "Dawn of the post-antibiotic era" (FALAGAS & BLIZIOTIS, 2007) there are an ongoing international debate regarding the terms multidrug resistance (MDR), extensive drug-resistance (XDR) and pandrug-resistance (PDR). Experts from the European Centre for

Disease Prevention and Control (ECDC) and the Center for Disease Control and Prevention (CDC) met to discuss these terms. It was then proposed that MDR is defined as non-susceptible to at least one agent in three or more antimicrobial categories. XDR is defined as non-susceptible to at least one agent in all but two or fewer antimicrobial categories, i.e. bacterial isolates remain susceptible to only one or two categories. PDR is defined as non-susceptible to all agents in all antimicrobial categories (MGIORAKOS *et al,* 2008).

Regarding the worldwide migration of the populations, control of these infections is also ever more difficult, not just in health care institutions, but also, for example, in children attending day care centers (HAWKEY & JONES, 2009).

2.3.1 Types of antibiotic resistance

Bacteria develop resistance to antimicrobials through a variety of resistance mechanisms that fall in three major categories: 1) antimicrobial target and receptor alteration, 2) antimicrobial modification or destruction and 3) prevention of the antimicrobial from reaching its intended target by either a decrease of permeability due to the LPS or the decrease of porins present in the OM (restricted to Gram-negatives) or overexpression of EPs that extrude the antimicrobial (VIVEIROS *et al*, 2010). These mechanisms can be intrinsic or acquired.

Innately resistant bacteria: Some species of bacteria, as an intrinsic property, have inherent resistance to two or more different classes of antibiotics. They are, thus likewise resistant to all members of those antibacterial classes. Intrinsic resistance is conferred mostly by drug impermeability through the cell wall. E.g. *P. aeruginosa* has intrinsic resistance to structurally unrelated antimicrobial agents and enterococci are resistant to cephalosporins (TENOVER, 2006; STRATEVA & YORDANOV, 2009).

Acquired resistance bacteria: Initially, susceptible bacteria become resistant to an antibacterial agent, under the selective pressure of the prolonged use of that agent (MACPHERSON *et al*, 2009).

This can be the result of spontaneous mutations – *vertical evolution* - in the existing DNA or acquisition of foreign DNA that encodes resistance – *horizontal evolution*. Respectively, it may occur in the chromosomal DNA or through transformation, conjugation or transduction (THOMAS & NIELSEN, 2005). The above-mentioned two types of acquired resistance give bacteria an extremely high genetic flexibility (SYKES, 2010).

Horizontal evolution is the main mechanism through which bacteria evolve into resistant organisms (HAWKEY, 2008a; SYKES, 2010; HAWKEY & JONES, 2009; HAWKEY, 2008b). Each of the mechanisms of horizontal evolution, which are conjugation, transduction and transformation, can introduce DNA with little or no homology with those of the recipient cell. These external DNA is obtained with the help of three major entities: plasmids, transposons and integrons.

Plasmids, which are linear or circular DNA molecules, found in both prokaryotes and eukaryotes are capable of autonomous replication. They can self-transfer between strains and species forming a mosaic-like structure with different resistant genes (HAWKEY, 2008b). Transposons are mobile DNA sequences that can migrate to different regions of the genome of bacteria or even to plasmids. There are two types of transposons. The Class I mobile elements or retrotransposons that are first transcribed to RNA and then again to DNA by reverse transcriptase enzyme; The Class II mobile elements, encode an enzyme called transposase, which regulates transposition (STRACHAN & READ, 2004). Integrons that are found in chromosomal DNA, plasmids and transposons have pieces of DNA called gene cassettes, which can be incorporated and expressed (SYKES, 2010; TENOVER, 2006).

Mechanisms of resistance

The most typical mechanisms of resistance are:

- Mutational alteration of the target protein: bacteria can become resistant through mutations that turn the target protein less susceptible to an agent. E.g. fluoroquinolone resistance is often due to alteration in the targets, i.e. DNA topoisomerases (NIKAIDO, 2009);

- Enzymatic inactivation of the drug: secretion of enzymes that degrade the drug are common against antibiotics of natural origin, e.g. through enzymatic phosphorylation, and against β-lactams, which are inactivated by β-lactamases (NIKAIDO, 2009);

- Acquisition of genes for less susceptible target proteins from other species: the production of mosaic proteins give bacteria more possibilities to avoid the noxious effect of antibiotics, e.g. penicillin resistance in *Streptococcus pneumoniae* is related to the production of mosaic proteins that in part came from other species (NIKAIDO, 2009);

- Prevention of drug access to targets: Drug access to a target can be reduced by an active efflux pump activity, e.g. Tet (K) encoded protein in *S. aureus,* or can be reduced locally. Local inhibition can be due, for example, to the action of Tet (M) and Tet (S) that by changing the ribosomal conformation can prevent the association of tetracyclines to ribosomes (ROBERTS, 2005). Another mean of preventing drug access to its target is the non-specific inhibition of drug access. Through decreased outer membrane permeability (only on Gram-negative bacteria), antibiotics cannot enter bacterial cells, e.g. *Enterobacter aerogenes* (NIKAIDO, 2001; NIKAIDO, 2009).

3. Absorption, Distribution, Metabolization and Excretion (ADME) system and transport mechanisms

Membrane transporters perform important functions for the cell such as providing nutrients, protecting the cell against noxious agents and establishing electrochemical gradients across membranes (ECKER & CHIBA, 2010).

The ADME kinetics of the organism, describes the disposition of a pharmaceutical compound within the organism, influencing thus the drug level and kinetics of drug exposure to the tissues. It is applicable to the pharmacokinetics and metabolism investigations in humans and animals, being thus critical in all phases of a fully integrated drug development program (www.netsci.org).

Efflux pumps are determinants for the absorption, distribution and excretion of drugs, toxic compounds and their metabolites (VAN BAMBEKE *et al,* 2000; HULS *et al*, 2009). During evolution, transport pathways emerged, allowing molecules, such as nutrients and metabolites, to cross cell membranes. As a result, the transport of small molecules is mediated by transmembrane proteins (ECKER & CHIBA, 2010). Numerous disorders caused by mutations in transporter genes underscore the physiological role of efflux pumps, such as Dubin-Johnson syndrome, sitosterolemia, or Tangier disease (ECKER & CHIBA, 2010).

4. Permeability barriers as bacterial defense systems

In order to survive, bacteria evolved a complex cell wall. This structure protects them against the exterior and permits the entrance of nutrients and extrusion of waste products (SILHAVY *et al,* 2010).

The first barrier to antibiotics in bacterial cells is the cell wall, which provides strength, rigidity and shape (VIVEIROS *et al*, 2007). The balance of membrane permeability is of great importance to bacteria because it limits the intracellular concentration of antibiotics (NIKAIDO, 2001).

4.1 Difference between Gram-positive and Gram-negative bacteria

Gram-negative cell wall:

In general, Gram-negative bacteria are more resistant to antibiotics because of the more complex architecture of their cell envelope, which includes an outer and inner membrane with the periplasmic space in between (SILHAVY *et al,* 2010) (Figure 1).

The outer membrane (OM) is a distinguishing feature of Gram-negative bacteria, because Gram-positives lack this structure. OM gives an additional protection from the environment by excluding toxic molecules and providing an additional stabilizing layer. It is a lipid bilayer that contains glycoplipids, mainly lipopolysaccharide (LPS). LPS is a virulence factor that typically consists of a hydrophobic domain known as lipid A or endotoxin, a non-repeating core oligosaccharide and a distal polysaccharide or O-antigen. The lipid A of LPS is responsible for the septic shock related to septicaemia, caused by these microorganisms (SILHAVY *et al,* 2010).

The outer membrane proteins (OMPs) of Gram-negatives are divided into two classes: lipoproteins and β-barrel proteins. The majority of the OMPs are transmembrane proteins. For example, OmpF is a transmembrane protein called porin that allows the passive diffusion of small molecules (SILHAVY *et al,* 2010).

As demonstrated in Figure 1, the periplasmic space is delimited by the OM and the inner membrane (IM). This structure gives Gram-negative bacteria a great evolutionary advantage because it entraps, for example, the potentially harmful molecules, such as degradative enzymes. In contrast to the Gram-positives, which lack this narrow space, Gram-negatives can pick toxic compounds here and expel them directly to the external medium through efflux pumps (represented by the vertical bold arrows in Figure 1), strongly reducing the number of molecules reaching their cytoplasmic targets. Within the periplasmic space there is the peptidoglycan whose main function is to give rigidity and shape to the cell wall. Peptidoglycan is made up of repeating units of the disaccharide N-acetyl

glucosamine –N-acetyl muramic acid which is cross-linked by pentapetide side chains. The amount of this molecule in Gram-negatives is much lower compared to Gram-positive bacteria (NIKAIDO, 2001; SILHAVY *et al,* 2010).

As widely known, bacteria do not have various organelles, that eukaryotic cells contain. These organelles perform a myriad of very important tasks to the cell wellbeing. In bacteria, all these "household tasks", like energy production, protein or lipid synthesis are performed in IM that is a phospholypid bilayer. Protein channels involved in the efflux mechanisms are also part of the bacterial cell envelope. Within the five superfamilies of transporters (chapter 5), MFS and RND are the most abundant. RND transporters (Figure 4), which are found so far exclusively in Gram-negative bacteria, are the major reason for antibiotic resistance (NIKAIDO, 2001).

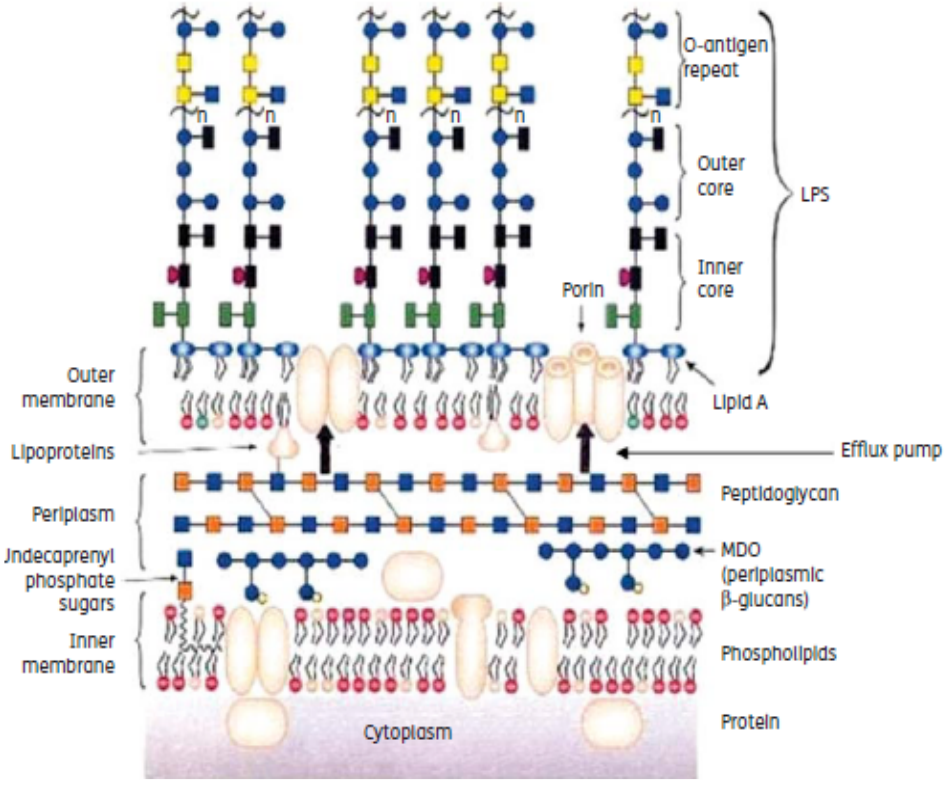

Figure 1- Schematic representation of the Gram-negative cell envelope

Gram-positive cell wall:

Gram-positives lack the OM, which is present in Gram-negatives; the peptidoglycan layer is thicker in Gram-positive bacteria, as shown by Figure 2. Interspersed in this layer, there are numerous polymers, called teichoic acids (TAs). This polymers account for 60 % of the mass of Gram-positives envelope, being thus major contributors to cell envelope structure and function (SILHAVY *et al*, 2010).

In addition to the TAs, there are proteins attached to the surfaces of Gram-positives. Some of these proteins have similar properties to those found in Gram-negative periplasmic space, furthermore the adhesion to extracellular noxious molecules is a task performed by these proteins (SILHAVY *et al*, 2010).

Because of their less complex cell envelope, Gram-positives, are considered much more susceptible to certain antimicrobials than Gram-negatives, which have been showing greater resistance to drugs (CORNAGLIA, 2009).

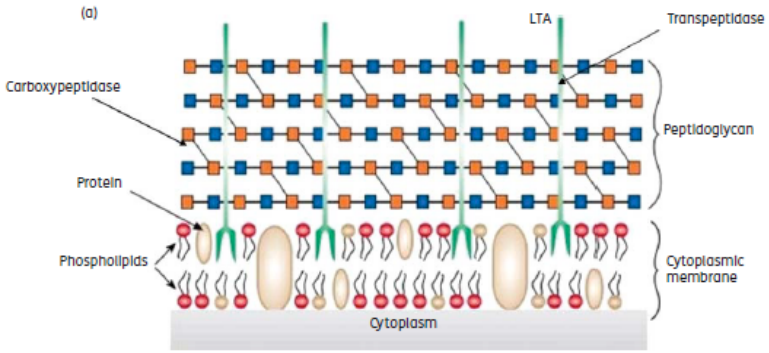

Figure 2 – Schematic representation of Gram-positive cell wall.

4.2 Transport systems (TENOVER, 2006)**:**

For most polar molecules, such as nutrients or metabolites, the lipid bilayer (Figure 1) of a cell membrane represents an impermeable membrane. The transport of small molecules is mediated by transmembrane proteins, whereas macromolecules and small particles cross membranes by various cytotic mechanisms (ECKER & CHIBA, 2010).

Diffusion is the translocation of molecules from a region of higher concentration to a region of lower concentration. This is mediated by pore forming channels. There are also membrane proteins that mediate the translocation of molecules that are too polar or too large to move across a membrane by diffusion. In contrast to pore forming channel proteins, carrier proteins bind their substrates at specific binding sites, resulting in the so called "facilitated diffusion" (ECKER & CHIBA, 2010). There are three types of membrane transporter proteins (ECKER & CHIBA, 2010) (Figure 3):

- Uniport, when the carriers mediate transport of a single substrate;
- Symport, when the carriers bind to different substrates and transport them together across the membrane;
- Antiport refers to those transporters that exchange one substrate for another across the membrane.

The so-called cotransport systems – symporters and antiporters - permit the simultaneous or sequential passive transfer of molecules or ions across biological membranes (Figure 3). Cotransporters are classified in symporters and antiporters depending on the direction of the second substrate. Symport refers to migration of molecules in the same direction, whereas antiport in opposite directions. Bmr and Blt from *Bacillus subtilis* are examples of antiporters (LI & NIKAIDO, 2009) whereas *E.coli* LacY functions as a symporter (GUAN & KABACK, 2006).

In contrast with the facilitated diffusion, active transport systems couple the transport with energy. There are two types of active transport. In primary active transport the energy comes from the ATP hydrolysis, whereas in the secondary active transport the energy is provided by the flow of ions, such as Na^+ (Sodium), H^+ (Hidrogen) or K^+ (Potassium).

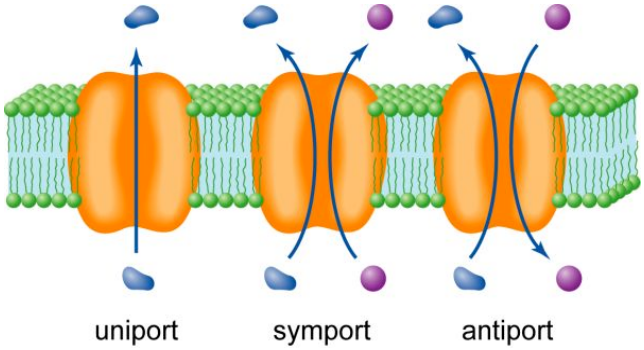

Figure 3 – Schematic representation of uniport, symport and antiport systems

The arrows represent the direction of transported ions or molecules through the transport system.

Source: vydavatelstvi.vscht.cz

5. Families of transporters

Efflux pump systems are present in all living systems, providing a frontline nonspecific defense, with standard responses to the external stimuli (RICE, 2007).

Substrate specific transporters constitute systems that are responsible for the extrusion of a given drug or class of drugs. On the contrary, the multidrug transporters are capable to handle a wide variety of structurally unrelated compounds. MDR transporters can be divided in two groups based on their structure and bioenergetics: 1) secondary multidrug transporters, that are predominant in bacteria, use the transmembrane electrochemical gradient of protons or sodium ions to extrude the drugs from the cell. Instead, the 2) primary multidrug transporters extrude their substrates with the energy of ATP hydrolysis. (ECKER & CHIBA, 2010; BORGES-WALMSLEY *et al*, 2003).

Although both primary and secondary transporters (Figure 5) are ubiquitous in bacteria, their relative presence seems to correlate with energy generation: fermentative bacteria tend to rely more

on the primary transporters while aerobic bacteria contain somewhat more secondary transporters in their genomes (PAULSEN *et al*, 2000***).***

Bacteria appear to be particularly endowed with multidrug resistance transporters. Considering predictions from the bioinformatic analysis of more than 200 available bacterial genomes, putative MDRs comprise 2 to 7 % of the total bacterial protein complement (SAIER & PAULSEN, 2001). Among these bacterial genomes, not all have been confirmed to be polyspecific, but quite a few of them are, underscoring the impressive resistance capacity of bacteria.

Functional studies and subsequent phylogenetic analysis demonstrated that bacterial MDR transporters could be organized into five evolutionary distinct protein superfamilies that significantly differ in bioenergetics, structure and transport mechanism (SAIER & PAULSEN, 2001). This structural and functional diversity gives bacteria a big potential to combat noxious agents (SAIER *et al*, 1998).

5.1 Primary multidrug transporters in bacteria:

5.1.1 ABC transporters in bacteria:

In bacteria, these transporters are predominantly involved in the import of essential compounds that cannot be obtained by diffusion (sugars, vitamins, metal ions and others). In spite of the importance of these transporters in eukaryotic cells, in bacteria their role is more limited. However, both antibiotic-specific and polyspecific ABC transporters have been identified (ECKER & CHIBA, 2010; NIKAIDO, 2009).

The most well-studied bacterial ABC multidrug transporters are LmrA and LmrCD, both from *Lactococcus lactis* (ECKER & CHIBA, 2010). LmrA is homologous to one half of the mammalian MDR1 (NIKAIDO, 2009; PUTMAN *et al,* 2000). Instead of the minor role played by LmrA in *L.*

lactis, its overproduction in *E. coli* confers resistance to cationic dyes, daunomycin and triphenylphosphonium (NIKAIDO, 2009).

The data available suggests that ABC transporters are more present in Gram-positive bacteria (ECKER & CHIBA, 2010). Another example of this superfamily of transporters is *S. aureus* Sav1866 that is homologue to LmrA (NIKAIDO, 2009; LI & NIKAIDO, 2009).

5.2 Secondary multidrug transporters

The majority of the multidrug efflux systems known to date are sensitive to agents that dissipate the proton motive force (PMF). Thus, these transporters couple the extrusion of drugs and other toxic agents with the exchange of protons or sodium ions. Related to their size and structures, secondary transporters can be subdivided into distinct superfamilies: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) superfamily, the resistance-nodulation-cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) superfamily (ECKER & CHIBA, 2010).

5.2.1 Major facilitator superfamily (MFS)

The MFS is the largest characterized superfamily of transporters (LI & NIKAIDO, 2009; FLUMAN & BIBI, 2009). These transporters are found in all living organisms and are implicated in the symport, antiport or uniport of various substrates, such as sugars, drugs, neurotransmitters or amino acids (FLUMAN & BIBI, 2009). This superfamily contains transporters that extrude one specific substrate and transporters that extrude a variety of them. Based on phylogenetic analysis, it appears that specific and MDR transporters in the MFS appeared randomly on the evolutionary tree, indicating that the broadening and narrowing of specificity toward particular drugs occurred repeatedly during evolution. For example, a single amino acid change in the *S. aureus* QacB

transporter enables it to recognize not only monovalent but also divalent cationic compounds (PAULSEN *et al*, 1996*)*. In addition, mutations that alter the substrate specificity of the transporters are thus of great importance as though, usually influence the transport of a subset of substrates. Therefore, these mutations usually occur in residues that participate in substrate binding. For example, Bmr, found in *Bacillus subtilis,* is one of the MFS transporters that harbor some of these residues, typically found inside of transmembrane domains (TMDs). Some of the substrate binding sites in this family are very large, thus with the ability to bind more than one substrates simultaneously (FLUMAN & BIBI, 2009).

These proteins can be clustered into two different groups, with either twelve or fourteen transmembrane segments (TMS). It has been proposed to function by an alternating access, rocker switch- type- structural mechanism, in which the substrate binding site is alternatively accessible from both sides of the membrane. For example, the EmrB of *E. coli,* where the MFS systems can function as components of tripartite systems (LI & NIKAIDO, 2009).

Other examples: NorA was first discovered in *S. aureus* and is from the 12-TMS cluster. It confers resistance to hydrophilic compounds and none to hydrophobic ones (FLUMAN & BIBI, 2009). Bmr, from *B. subtilis*, is structural and functionally homolog to NorA (AHMED *et al* 1995).

5.2.2 Small multidrug resistance superfamily (SMR)

The SMR superfamily, most found in Eubacteria (BAY *et al*, 2008), is the smallest secondary MDR transporter known, composed of around 100 amino acids. SMR transporters are believed to span the cytoplasmic membrane as four transmembrane (TM) α-helices with short hydrophilic loops, thus making them highly hydrophobic.

Members of this superfamily have been identified, encoded by a variety of plasmids and transposable members giving high resistance to a wide range of antibiotics. E.g. QacH in *Staphylococcus saprophyticus*. (PUTMAN *et al*, 2000).

Among Gram-negative bacteria, EmrE from *E. coli*, is considered the structural archetype of all SMR proteins (SCHULDINER *et al*, 2001). The scientific data available reveals that these transporters function as an oligomer, most likely a dimer. There are other examples, like the Mmr of *Mycobacteria tuberculosis* that confers resistance to ethidium bromide, erythromycin, or the antiseptic resistance multidrug transporter QacE present in *Klebsiella aerogenes* (PUTMAN *et al*, 2000).

5.2.3 Resistance-Nodulation-Cell Division Superfamily

The RND transporters (Figure 4 and Figure 5) are responsible for the high intrinsic antibiotic resistance found in Gram-negative bacteria (MURAKAMI, 2008). They are also found in Gram-positive bacteria as well as in *M. tuberculosis* where one of these RND type pumps is responsible for the efflux of the first-line antimycobacterial drug, isonazide (PASCA *et al*, 2005). These transporters are composed of approximately 1000 amino acid residues, adopting a 12-helical structure, comprising large periplasmic or extracytoplasmic domains between helices 1 and 2 and between helices 7 and 8.

RND transporters contain an astonishing wide range of substrate specificity surpassing even the ABC transporters (ZGURSKAYA & NIKAIDO, 2002). These pumps require three units: a transporter protein in the IM, called AcrB, in association with two more classes of proteins, such as the OMF (outer membrane factor) Tol C and the MFP (membrane fusion protein) AcrA from *E. coli* (Figure 4) (MURAKAMI, 2008). These transporters are very complex because without one of the three parts available, the complex turns non-functional.

It was shown that by inactivating the AcrB, bacteria become susceptible to some drugs (LI & NIKAIDO, 2009). Based on former results it can be supposed that the periplasmic loops of RND transporters are critical in substrate determination, possibly containing multiple binding sites for structurally unrelated compounds (ECKER & CHIBA, 2010; MURAKAMI, 2008).

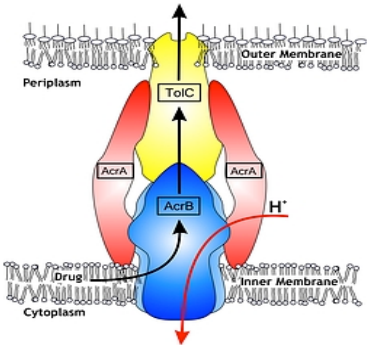

Figure 4 – Schematic representation of the RND superfamily: the structure of AcrAB-Tol C (POS, 2009**).**

The tripartite complex is represented by the different colours: in blue, the inner membrane protein AcrB, in red the membrane fusion protein AcrA and in yellow the outer membrane factor TolC.

5.2.4 Multidrug and Toxic Compound Extrusion Superfamily (MATE)

There are twenty MATE transporters characterized to date. Most members of this family consist of 400-500-residue polypeptides with 12 putative transmembrane helices (TMHs) (LI & NIKAIDO, 2009). Phylogenetic analyses classify these transporters into three subfamilies. Little is known about the molecular mechanism underlying their organic cation transport. However, there are some similarities with the MFS superfamily (LI & NIKAIDO, 2009; MCALEESE *et al*, 2005).

Examples: NorM from *Vibrio parahaemolyticus* and MepA from *S. aureus* are both MATE transporters implicated in the resistance to antibiotics, conferring resistance to fluoriquinolones and tigecycline, respectively (LI & NIKAIDO, 2009; MCALEESE *et al*, 2005).

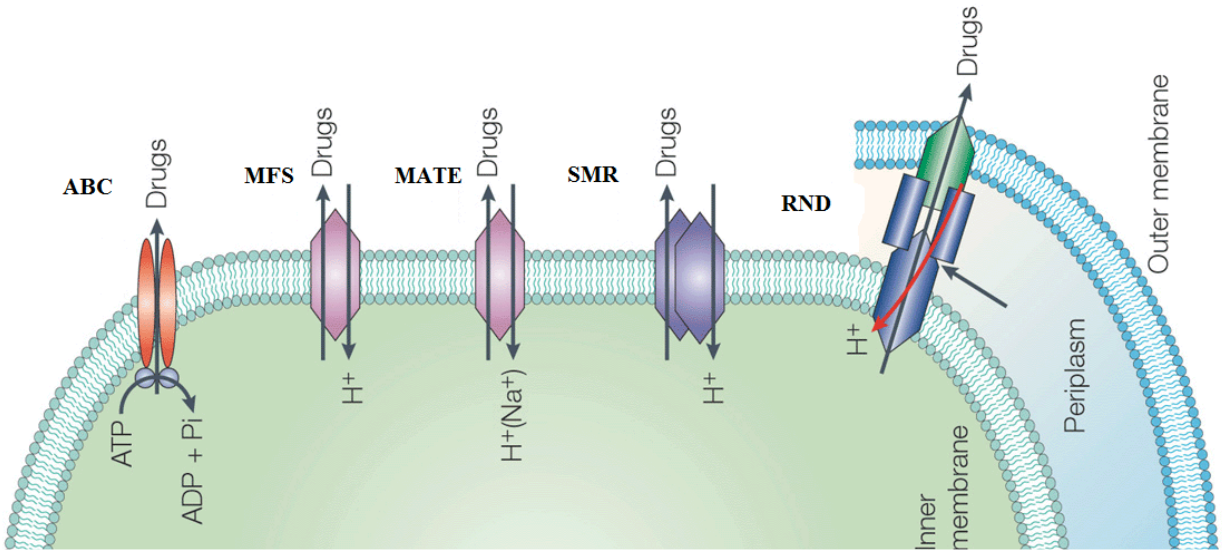

Figure 5 – Schematic representation of the five superfamilies of multidrug transporters.

The ATP-binding cassette (ABC) superfamily is the only one belonging to the primary multidrug transporters. The other four superfamilies belong to the secondary multidrug transporters.

Source: www.nature.com

6 – Multidrug resistance in cancer

Cancer is one of the most fatal diseases worldwide (WHO). In poor and developed nations, cancer is a leading cause of death. 70 % of all cancer deaths occurred in middle and low-income countries. The WHO projections for 2030 are around 11.5 million deaths. Limited improvement in diagnosis, surgical techniques, patient care and adjuvant therapies are among the top causes of this projection (FIEDLER, 2002)

6.1 Cancer burden

In Europe, 3.2 million people are diagnosed with cancer every year (FERLAY *et al*, 2010). In both sexes, in EU, cancers with the higher rates of incidence are: prostate cancer, breast cancer, colorectal cancer and lung cancer. Lung and breast cancers have the highest mortality rates (Figure 6).

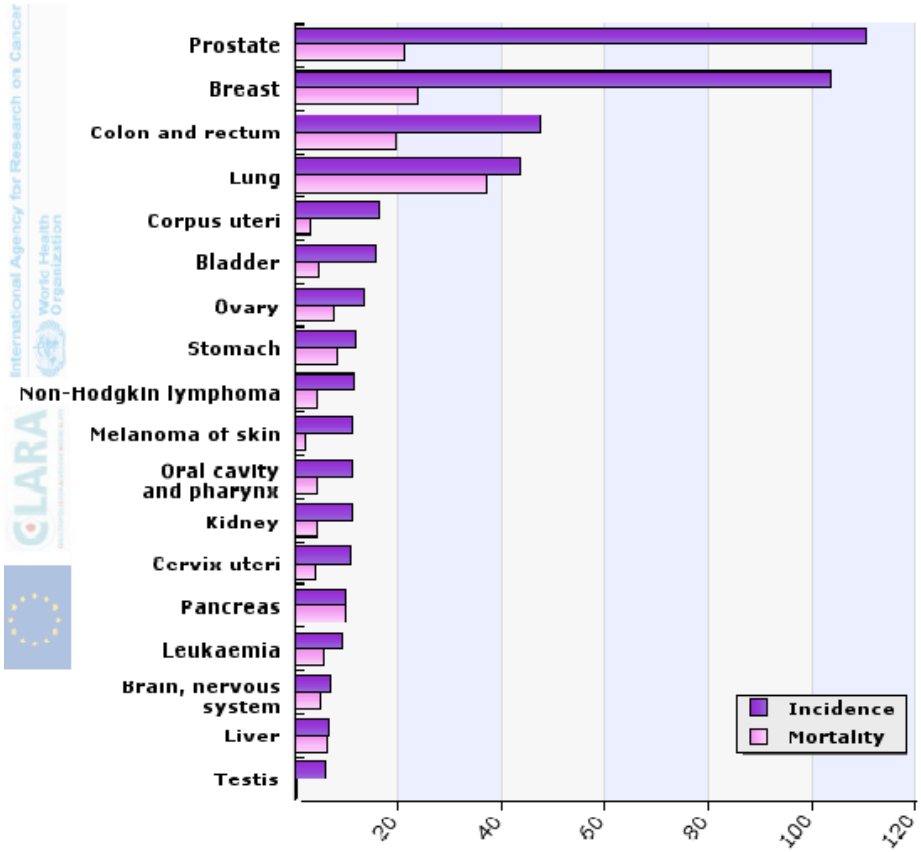

Figure 6. 2008 statistics of the cancer incidence and mortality rates in EU for both sexes.

Source: http://www.iarc.fr/

There are also differences in the incidence between men and women. In men the types of cancer with higher mortality rate are: prostate cancer, lung cancer, and colorectal cancer. In women, the most common types of cancer are breast cancer, colorectal cancer and lung cancer (FERLAY *et al,* 2010).

6.2 Causes of cancer

The transformation from a normal cell into a cancer one is a multistage process that begins from one single cell (STEVENS & LOWE, 2000; LUO & ELLEDGE, 2008). These changes can be due to genetic factors, ageing and three categories of external agents (www.cancer.org) such as:

- Physical carcinogens (e.g UV light);
- Chemical carcinogens (e.g tobacco smoke);
- Biological carcinogens (e.g HPV virus).

7. Relationship between bacterial infection and cancer

Bacterial infections can also be risk factors for cancer development, for instance *Helicobacter pylori* and *Salmonella typhi* can be involved in cancer development. *H. pylori* infects, at least 50 % of worldwide population. Within other malignancies, such as chronic inflammation or peptic ulcer disease, it is a risk factor for gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. In order to combat *H. pylori* an initial treatment has been developed consisting of a triple therapy that contains a proton pump-inhibitor (PPI). The treatment consists of clarithromycin, metronidazole or amoxicillin with a PPI, such as esomeprazole (BERGMAN & D'ELIOS, 2010; FISCHBACH *et al*, 2009). Resistance to clarithromycin has yet been reported in various countries (FISCHBACH *et al,* 2009).

Chronic infection with *S. typhi* can be related to gallbladder cancer. Carriers of this pathogen have an eight-fold higher risk of developing this type of cancer than non-carriers. The exact pathogenesis is yet to be understood but there is increasing evidence that the products of the degradation of bile-salts may contribute to tumorogenesis (SAMARAS *et al*, 2010).

8. Carcinogenesis - the development of cancer

In the human body, cells grow and divide in a controlled fashion to produce more cells, in order to keep the body healthy. Certain stimuli cause changes in genetic material that result in permanent alteration of the normal cellular growth pattern (STEVENS & LOWE, 2000). These distorted cells fail to respond normally to signals controlling cell growth; they are termed neoplastic (STEVENS & LOWE, 2000), because they proliferate uncontrolled, forming a tissue called neoplasm, which means "new growth".

As shown by Figure 7, there is a difference between the normal and cancer cell division. Apoptosis or programmed cell death is a normal component of the development and health of multicellular organisms. Through apoptosis cells die in a controlled and regulated manner.

The main reason for the difficulties in cancer therapy is that cancer cells can spread to other organs. This process is called metastasis and refers to the growing of a secondary tumor that was originated from a primitive neoplasm (GUPTA & MASSAGUE, 2006).

As seen in Figure 8, the invasive cancer cells migrate from the primary tumour site into the surrounding tissue through blood vessels. Cells are thus carried to distant organs where they lodge in their small capillaries. They form secondary tumours by extravasating to the surrounding tissue (STEVENS & LOWE, 2000). As neoplastic cells are continually growing, they need an appropriate set of support tissues, particularly an adequate set of vascular supply that is why angiogenesis is essential for the growth of neoplasms (SCHEIDER *et al*, 2009).

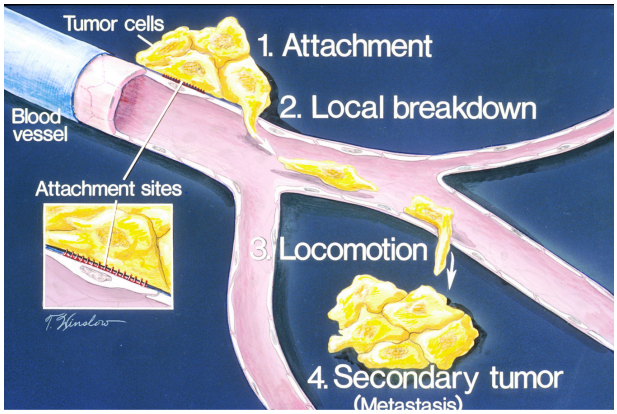

Figure 8. Schematic representation of the metastasizing process.

1 – Attachment of the tumour cells; 2 – Influx of tumour cells through a vessel breakdown; 3 – Locomotion of the tumour cells through the circulatory system; 4 – Adhesion of the tumour cell mass in a different body location.

Source: http://trendsupdates.com

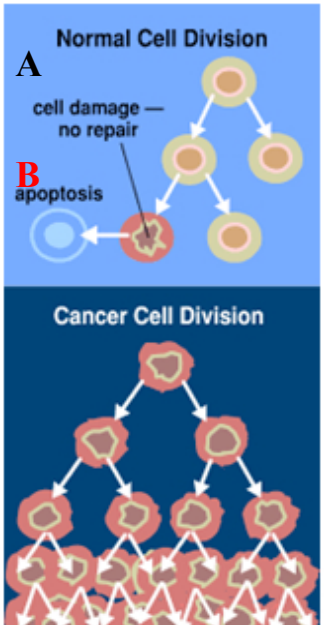

Figure 7. Cellular division in normal and cancer cells.

A – Normal cell division; B – Cancer cell division;

Source: http://www.jyi.org

9. Cancer therapy and the problem of resistance mechanisms to anticancer agents

Chemotherapy is the treatment of choice for ~50 % of all types of cancer (AMBUDKAR *et al,* 2005), the effectiveness of chemotherapy – a cancer treatment that uses drugs to destroy cancer cells - is of big concern for medicine. Combination chemotherapy is the use of different chemical agents simultaneously. It was first introduced in 1963 for the treatment of resistant cancers (SHEPS & LING, 2007).

9.1. Main groups of anticancer drugs

Among the great amount of cells that forms a neoplasm, not all are in the same development stage or respond in the same way to the same stimulus. In order to prevent selection of resistant cancer

cells, contemporary chemotherapy uses combinations of various drugs of different targets. There are five main families of anticancer drugs:

- **Alkylating agents:** drugs that bind to the DNA, e.g. cisplatin, ifosfamide (STAVROVSKAYA, 2000; TASCILAR *et al,* 2007);

- **Anticancer antibiotics:** These agents act by topoisomerase inhibition, e.g. daunorubicin, doxorubicin (STAVROVSKAYA, 2000; KURUVILLA, 2009);

- **Antimitotic agents:** Depolymerization of microtubules and damage to mitotic spindle, e.g. vinca alkaloids and taxanes (STAVROVSKAYA, 2000; PEREZ, 2009);

- **Antimetabolites:** The main mechanism of action of these agents is the inhibition of enzymes participating in DNA or RNA synthesis, e.g. methotrexate, gemcitabine (STAVROVSKAYA, 2000; MERL *et al,* 2010).

- **Hormones:** Hormones can be a natural product of an organ or represent abnormal synthesis reflecting unregulated cancer cell metabolism. Drugs, such as tamoxifen are antagonists of the estrogen receptor, obstructing the effect of the hormones (JORDAN, 2006).

9.2. Resistance to anticancer agents:

In order to resist to the effects of anticancer drugs, tumour cells have various ways to resist their noxious effects. There are thus four main mechanisms of drug resistance:

- **Decrease of drug accumulation by the cells:** drugs inside the cells can be extruded due to the activation of transporter proteins. Otherwise, these drugs remain inside the cells, able to exert their toxic effect. E.g. P-gp. (STAVROVSKAYA, 2000; LAPENSEE & BEN-JONATHAN, 2010).

- **Detoxification of the drug in the cell:** Cells have mechanisms to turn drugs ineffective. One of these mechanisms is the cellular glutathione system (CGS). Glutathione forms a conjugate with the reactive site of the drug that is more water-soluble and less active. This conjugate is then extruded from the cell through transporter proteins, such as GS-X (including MRP) (STAVROVSKAYA, 2000; MOSKAUG *et al,* 2005).
- **Alteration of drug targets:** Gene mutations are common in cancer cells that often render drug targets unrecognizable. Sometimes, during the progression of cancer, some targets are lost, for example the estrogen receptor in the progression of breast cancer. Another way of drug target alteration is the increase of target proteins, which is caused by the over-expression of the gene that controls the target molecule (STAVROVSKAYA, 2000; LO & SUKUMAR, 2008).
- **Key genes that control apoptosis:** One of these genes is p53, also known as "guardian of the genome". p53 is often altered in tumour cells, resulting in impaired function and inducing apoptosis (STRACHAN & READ, 2004; STAVROVSKAYA, 2000; BELL & RYAN, 2007).

10. The importance of ABC transporters in cancer

Multidrug resistance is a major obstacle to the success of cancer chemotherapy. It is mainly related to the expression of ABC transporters (HALL *et al,* 2009). Forty-nine members of this superfamily have already been identified and classified in seven subfamilies based on their phylogenetic similarity (KLEIN *et al,* 1999).

P-glycoprotein (P-gp), the product of *MDR-1 (ABCB1)* gene, was the first to be discovered and is the most known efflux pump within ABC transporters (HALL *et al,* 2009). ABCB1 is found in various resistant tumour cell lines and is also naturally expressed in many tissues (MARCHETTI *et al,* 2007).

10.1 Physiological localization of ABCB1 (P-glycoprotein)

As seen in Figure 9, ABCB1 is expressed in the lumen of the endothelial cells, which comprises the blood-brain barrier. It is found in the canalicular membrane of hepatocytes, brush-border membrane of proximal tubules in the kidney, mediating thus the expression into the urine. ABCB1 also prevents the absorption of substrates into the human body because it is present in the intestine epithelial cells. ABCB1 is also present in the syncytiotrophoblasts and bone marrow (HULS *et al,* 2009).

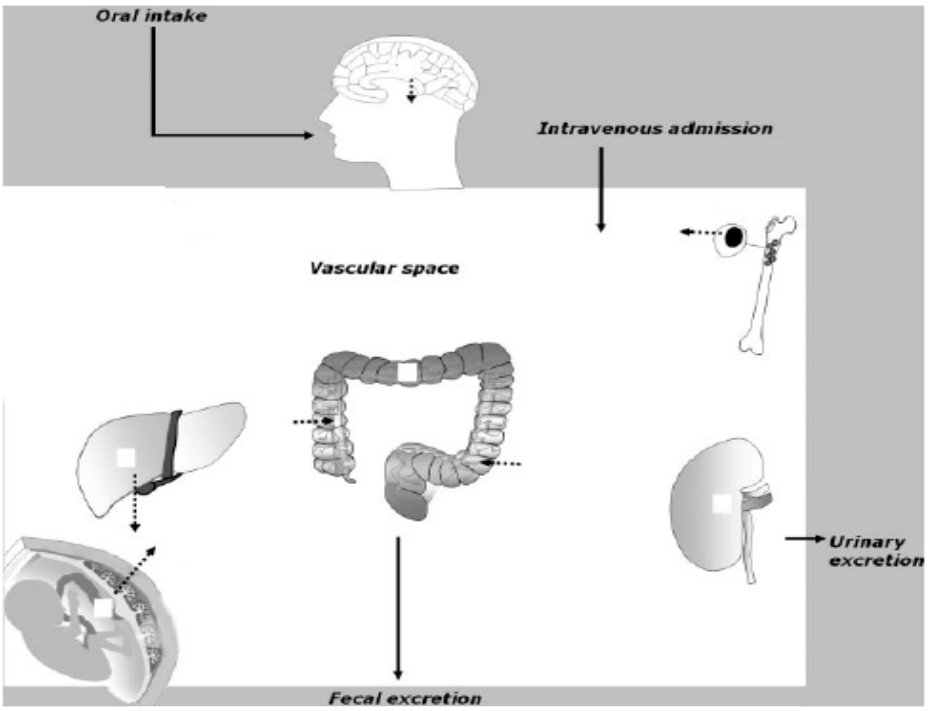

Figure 9. Physiological distribution of ABCB1 (P-glycoprotein) (HULS *et al,* 2009**).**

Representation of the various organs where ABCB1 has a normal physiological role.

In humans, defective ABC transporters are associated with several diseases, including Dubin-Johnson syndrome, sisterolemia and Tangier disease (ECKER & CHIBA, 2010). ABC transporters are often responsible for clinical MDR; consequently these transporters are also often a sign of poor

prognosis (STAVROVSKAYA & STROMSKAYA, 2008). Mutations in genes encoding ABC transporters can induce a multitude of defects, presenting as autosomal recessive conditions. A good example is the cystic fibrosis transmembrane regulator protein (CFTR), a member of the ABCC subfamily. A mutation in the encoding gene on chromosome 7 impairs the synthesis of CFTR resulting in cystic fibrosis (LEONARD *et al,* 2003).

Like MRP1 – the second drug transporter in humans (COLE *et al,* 1993) – ABCB1 is thought to provide protection to normal tissues, preventing the accumulation of or exposure to toxic substances (HOLLAND *et al*, 2003; LEONARD *et al,* 2003). Mice lacking ABCB1 homologue (mdr1a and mdr1b) have a subtle phenotype indicating a role for ABCB1 in the physiological defense against xenotoxins (SCHINKEL, 1997).

10.2 Structure of ABC transporters

In contrast to prokaryotes, in eukaryotes the major mechanism of efflux is dependent on proteins that derive their transport energy from the hydrolysis of ATP. The majority of these transporters belong to the ABC superfamily (BORGES-WALMSLEY *et al,* 2003), such as ABCB1 (also known as P-gp), ABCC1 [also known as multi-drug resistance-associated protein (MRP1)] or ABCG2 [also known as breast cancer-resistance protein (BCRP)] (LIU, 2009).

The members of this superfamily translocate a wide range of substances (including sugars, amino acids, sterols, peptides, antibiotics, xenobiotics between others) across cytoplasmic and organellar membranes (LEONARD *et al,* 2003).

In eukaryotes, molecules are mostly transported from the cytoplasm to the outside of the cell or into an intracellular compartment (endoplasmic reticulum, mitochondria, peroxisome) (DEAN *et al,* 2001).

The topology of these transporters indicates the existence of highly hydrophobic transmembrane domains (TMDs) with between 4 and 10 TM α-helices – typically six per domain - and hydrophilic

cytosolic ATP binding/hydrolysis domains, the so called nucleotide binding domains (NBDs). The minimal structural requirement for an active eukaryotic ABC transporter is thought to consist of two TMDs and NBDs each (Figure 10) (LIU, 2009; TUSNÁDI *et al, 1997*).

All ABC proteins, in prokaryots and eukaryotes, have two cytoplasmic nucleotide binding domains containing conserved sequence motifs for binding/hydrolysis of ATP (LOCHER & BORTHS, 2004). In eukaryotes, the NBD's contribution to the protein activity is often non-equivalent – deletion of either NBD abolishes transport (JHA *et al,* 2003) - whereas in some prokaryotes both are functionally identical. The primary structures of NBD's show ~25% sequence identity across the whole superfamily (KERR, 2002). Marked sequence conservation is observed over three short regions found in NBDs: the Walker A and Walker B regions, which are separated by approximately 90-120 amino acids and between which lays the signature motif (Figure 10) (DEAN *et al,* 2001).

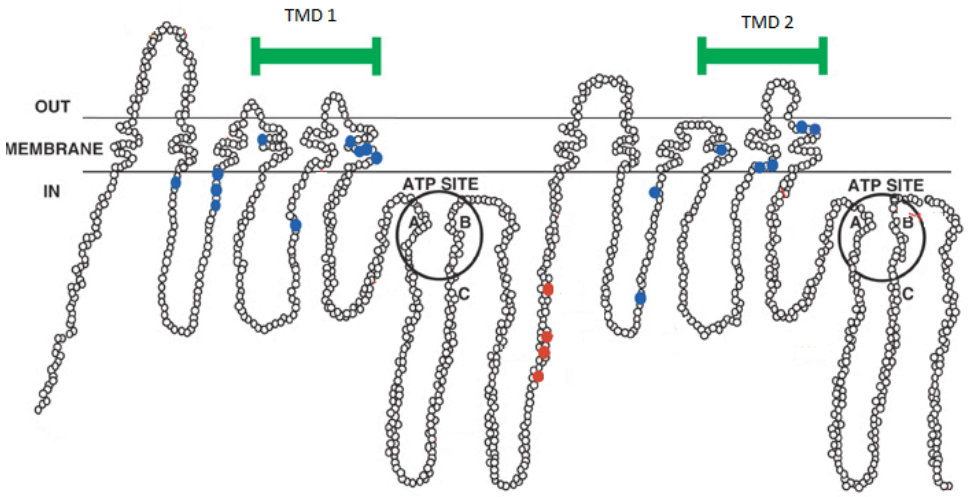

Figure 10. Schematic representation of the organization of the P-glycoprotein.

In this 2-D model of P-gp each circle represents an amino acid residue, the blue circles represent mutations that alter substrate specificity, and the red circles represent phosporylation sites. The Walker A, B regions and the signature motif or C region are circled.

Adapted from Reference *AMBUDKAR et al,* 2003.

10.2.1 Structure of ABCB1

ABCB1 comprises 12 transmembrane segments divided into two TM domains, each linked with an ATP-binding domain (Figure 10).

When a substrate approaches the ABCB1, it binds to the high affinity binding domain, promoting the ATP hydrolysis, resulting in a conformational change that shifts the substrate to a lower affinity binding site, releasing it into the extracellular space or outer leaflet of the membrane. ABCB1 is able to bind substrates again after ATP hydrolysis at the second binding site (LEONARD *et al,* 2003).

11. Efflux pump inhibitors

11.1 Efflux pump inhibitors in bacteria

The use of efflux pumps inhibitors (EPIs) has been investigated in order to improve and potentiate the activity of antibiotics. The goal has been the development of compounds that decrease the level of intrinsic resistance, significantly reverse acquired resistance, and promote the decrease of the frequency of emergence of multidrug resistant bacteria (LOMOVSKAYA *et al,* 2001). One such example is the EPI carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that promoted the inhibition of EB in *E.coli* AG 100_{TET} (*E.coli* with overexpressed AcrAB efflux pump) as shown by Martins *et al* (MARTINS *et al,* 2009). Various EPIs may be the substrates for the pumps that they inhibit (LI & NIKAIDO, 2009). Representative efflux pumps such as the *E. coli* AcrAB-TolC have been used to screen and characterize possible EPIs. Thus, one of the most critical issues in the quest for new EPIs is the understanding of how EPIs block the transport of drugs out of the cell, as shown by Table 2 (LI & NIKAIDO, 2009).

Table 2. Examples of bacterial EPIs, their targets and antibacterials with enhanced activity (LI & NIKAIDO, 2009**).**

EPIs	**Gram-negative bacteria**	
	Efflux pumps targeted	**Antibacterials with enhanced activity**
Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)	Secondary transporters	Multiple antibacterials
Arylpiperazines: 1-(1-naphthylmethyl)-piperazine and others	RND pumps of *A. baumannii*, *Citrobacter freundii*, *E. aerogenes, E. coli, K. pneumoniae, V. cholerae*	Fluoroquinolones, tetracyclines, and others
Quinoline derivatives	AcrAB-TolC of *E. aerogenes*, *E. coli*, *K. pneumoniae*	Multiple antibacterials
Extracts of *Berberis aetnensis*	*E. coli*, *P. aeruginosa*, *S. aureus*	Ciprofloxacin
EPIs	**Gram-positive bacteria**	
Fluoroquinolone derivatives	MepA and NorA of *S. aureus*	Fluoroquiniolones
GG918 (synthetic)	*S. aureus*	Fluoroquinolones
Reserpine	Bmr of *B. subtilis*, EfrAB of *E. faecalis*, NorA and Tet(K) of *S. aureus*, PmrA and PatAB of *S. pneuomoniae*	Fluoroquinolones and tetracyclines

Concerning the fact that one single bacterium has various efflux pumps conferring resistance to various antibiotics, it seems obvious to develop broad-spectrum inhibitors, capable to interact with various efflux pumps (POOLE, 2007). Pagès *et al* resumed the possible targets of bacterial inhibitors, such as collapsing energy used by efflux pumps, inhibiting their functional assembly or the blockage of the outer membrane channel (PÀGES & AMARAL, 2009).

11.2 Efflux pump inhibitors in eukaryotes

ABCB1 demonstrates high transport capacity and a wide range of substrate specificity. In general these transported molecules are hydrophobic and amphipathic, uncharged or basic, although negatively charged molecules can also be transported (COLE *et al,* 1992).

The most common approach to interfere with overexpressed ABCB1 activity is the use of inhibitors (YANG *et al,* 2008). Until now three generations of ABCB1 inhibitors have been identified and developed. Some ABCB1 substrates and inhibitors are demonstrated by Table 3.

Table 3. ABCB1 substrates and inhibitors (MARCHETTI *et al,* 2007; LEONARD *et al,* 2003).

ABCB1 substrates	ABCB1 inhibitors
Adriamycin	Verapamil
Daunorubicin	Cyclosporin A
Epirubicin	Valspodar
Paclitaxel	Quinidine
Docetaxel	Omeprazole
Vincristine	Tamoxifen
Vinblastine	Tariquidar
Etoposide	Zosuquidar trihydrochloride
Mitoxantrone	Biricodar
Actinomycin D	Elacridar

It has been established that ABCB1 is capable to interact with more than 200 compounds, that can be classified based on the transported substrates and the modulators (DIDZIAPETRIS *et al,* 2003).

In cancer cells, ABCB1 is associated with the MDR phenotype, mediating resistance to anthracyclines, vinca alkaloids, colchicine, epipodophyllotoxins and paclitaxel (LEONARD *et al,* 2003). Thus, the wide spectrum of resistance and the poor prognosis urged the need to circumvent

the actions of ABCB1. Broad spectrum inhibitors appeared in the 1980's and brought a certain degree of optimism; these molecules could thus restore some sensitivity to chemotherapy (ECKER & CHIBA, 2010). The use of established drugs for the modulation of ABCB1 function formed the first generation of inhibitors (TSURUO *et al,* 1984).

So far, three generations of compounds have been identified and developed as ABCB1 inhibitors that can be also classified as competitive and non-competitive inhibitors. As the name implies, the competitive ones fight with the cytotoxic agents for transport. If successful, the cytotoxic agent could be transported by the pump, remaining inside the cell. Their non-competitive counterparts do not compete for the same target. They bind to another target, in order to change the pump conformation, so that the active site is no longer recognized by the substrates (YANG *et al,* 2008).

FIRST GENERATION of inhibitors

Verapamil and tamoxifen are among the most known ABCB1 modulators that belong to the first generation of compounds, which means that, they are not specific for ABCB1. In general they are less effective and toxic (MARCHETTI *et al,* 2007; YANG *et al,* 2008).

SECOND GENERATION of inhibitors

The approach used for the second generation of ABCB1 inhibitors involved the use of chemical derivatives of the drugs, in order to improve potency, specificity and ABCB1 binding affinity. They are divided in two categories, those that are analogues of the first generation, i.e.dexverapamil (WILSON *et al,* 1995) and those with new chemical structures, i.e. biricodar (YANG *et al,* 2008). In comparison with the first generation of inhibitors, inhibitors of the second generation have higher ABCB1 inhibitory capacity and less toxic side effects; however they can have unpredictable interactions between anticancer drugs (YANG *et al,* 2008).

THIRD GENERATION of inhibitors

Concerning the strategies used in the third generation of compounds, the goal is to overcome the toxicity of the previous generations to improve the pharmacokinetic interactions of the compounds. The new tools at disposal, such as computational chemistry or molecular pharmacology, have been fundamental for shifting the approach of blind screening to rational drug design. These new tools, also with the help of quantitative structure-activity relationship (QSAR) have been very useful for the discovery of compounds, such as GF120918 (Elacridar) (YANG *et al,* 2008).

12. Hydantoin derivatives and their pharmacological use

12.1 Physiological and biochemical role of hydantoins

Hydantoins participate in the purine catabolic pathway that regulates the purine pool in the cell to provide precursors for nucleic acid synthesis, hydantoinases, hydantoin transporters (SUZUKI & HENDERSON, 2006).

Hydantoin is a heterocyclic organic compound, the product of glycolic acid and urea, also known as glycolyurea. In a more general sense, hydantoins refer to chemical compounds that have substituent groups bonded to a hydantoin ring.

Hydantoin derivatives are famous for their physiological activity as anticonvulsants, which explain the continuous research interest on this class of compounds (AHMEDOVA *et al,* 2009). Epilepsy is widely recognized as one of the most known neurological diseases in man. Its most known characteristic is the uncontrolled convulsions, the result of excessive temporary neuronal discharges. 5,5-diphenylhydantoin, commonly known as phenytoin, is used since 1938, being the treatment of choice for generalized tonic-clonic seizure, instead of a high number of side effects related (THENMOZHIYAL *et al,* 2004).

They are also widely used in numerous pharmacological applications. Thus, many derivatives have been identified as anticarcinogenics, antimuscarins, antiulcers, antiarrythmics, antivirals, antidiabetics, serotonin and fibrinogen receptor antagonists, inhibitors of the glycine binding site of the NMDA receptor and antagonists of leukocyte cell adhesion (OLIMPIERI *et al,* 2009) and other activities including fungicidal and anti-HIV activity (KHANFAR *et al,* 2009).

In general, all these activities are possible because of the interaction between the hydantoin ring (shown in Figure 11) and the different substituent groups attached to it (OLIMPIERI *et al,* 2009). It is well established that the type of substituent at 5-position in the hydantoin ring is of crucial

importance for the pharmacological action of the corresponding compounds (AHMEDOVA *et al,* 2009).

12.2 Structure of hydantoin derivatives

As summarized by Roszak and Weaver (ROSZAK & WEAVER, 1998) the hydantoin ring can be split into two structural fragments responsible for the anticonvulsant activity and for the mutagenic effects, the two competing biological effects, as shown in Figure 11. (AHMEDOVA *et al,* 2009)

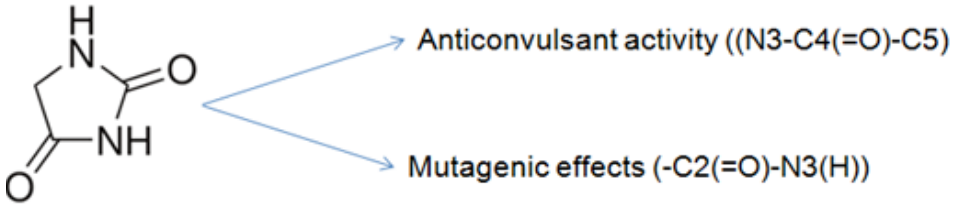

Figure 11. Structure of the hydantoin ring and its double activity
(AHMEDOVA *et al,* 2009**)**

II - OBJECTIVES OF THE THESIS

The present work involves the study of primary and secondary efflux pumps, in eukaryotic and prokaryotic cells, respectively. In bacteria, the experimental models were *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *E. coli* AG 100 and *Salmonella* Enteritidis NCTC 13349 strains. In the cancer model, parental mouse T-cell lymphoma cell line and its human *MDR1*(*ABCB1*)-gene transfected subline were used.

The main aim of the study is to look for new effective, hydantoin modulators of efflux pumps in bacteria and cancer cells to develop new pharmacological agents that reverse multidrug resistance.

The main goals of the study:

The biological activity of hydantoin derivatives in bacteria will be demonstrated as follows:

1. Determination of minimum inhibitory concentrations of the hydantoin derivatives on different bacterial strains;
2. Real-time accumulation of ethidium bromide by different bacterial strains in the presence of hydantoin derivatives using real-time fluorometry.

The biological activity of hydantoin compounds in cancer cells will be demonstrated by different methods:

1. The antiproliferative and cytotoxic effect of the compounds will be studied in parental (PAR) mouse T-lymphoma cell line and its human *MDR1*-gene transfected subline (MDR) by MTT (dimethyl thiazolyl blue tetrazolium bromide) test.

2. Screening of possible effective MDR modulators by real-time fluorometry assessing accumulation of EB on a real-time basis in PAR and MDR mouse T-lymphoma cells.

III – MATERIALS AND METHODS

1 - Materials

1.1 Compounds tested

Thirty hydantoin derivatives (**SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, P10, P11, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, KK-XV, Thioam-1, JHF-1, JHC-2, JHP-1, Fur-2, GL-1, GL-7, GL-14, GL-16**, **GL-18)** were kindly provided by Dr. Jadwiga Handzlik and Prof. Dr. Katarzyna Kieć-Kononowicz, Cracow, Poland). The structures are confidential. All the compounds were dissolved in DMSO (Merck, Darmstadt, Germany).

1.2 Solutions

Table 4. Used solutions

Solutions	**Preparation of stock solutions**
PBS[1] (Phosphate-Buffered Saline)	Tablet, containing [137 mM NaCl; 2.7 mM KCl; 8 mM Na_2HPO_4; 1.5 mM KH_2HPO_4 (pH 7.4)]
EB[1] (Ethidium Bromide)	10 mg/mL in sterile bi-destilled water (kept in the dark)

[1] Sigma (St. Louis, MO, USA)

MTT (Thiazolyl blue tetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA) and a stock solution of 5 mg/mL was prepared in PBS.

SDS (sodium dodecyl sulphate) purchased form Sigma-Aldrich Chemie GmbH (Steinheim, Germany) was used to prepare a 10% SDS solution in 0.01 M HCl.

1.3 Bacterial strains.

The following bacteria were employed in this study: *Salmonella* Enteritidis NCTC 13349, wild-type *Escherichia coli* K-12 AG100 (*argE3 thi-1 rpsL xyl mtl* delta (*gal-uvrB*) *supE44*) (OKUSU e*t al,* 1996), *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. *E. coli* K-12 AG100 have been previously characterized by Okusu *et al* (OKUSU e*t al,* 1996).

Stocks of bacteria were kept at -80 ºC in liquid media, supplemented with 15% (v/v) glycerol.

1.4 Cell lines.

L5178Y mouse T-cell lymphoma cells (ECACC cat. no. 87111908, U.S. FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus, as described previously (PASTAN *et al,* 1988; CHOI *et al,* 1991). The *ABCB1*-expressing cell line was selected by culturing the infected cells with 60 ng/mL of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to maintain the MDR phenotype. L5178 mouse T-cell lymphoma cells (parental, PAR) and the human *ABCB1*-gene transfected sub-line (MDR) were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine (Lonza BioWhittaker) and antibiotics (penicillin, streptomycin) at 37°C and in a 5% CO_2 atmosphere.

1.5 Broths and culture media.

All the solutions listed in Table 6 were dissolved in distilled water. The solutions were sterilized and autoclaved for 15 minutes, at 121 ºC.

Table 5. Media

Bacterial culture media	**Preparation /Composition** (per liter)
TSB[1] (Tryptic Soy Broth)	30.0 g of TSB (17 g tryptone, 3 g soytone, 2.5 g di-potassium hydrogen phosphate, 5 g sodium chloride, pH 7.3 ± 0.2 at 25 ºC)
TSA (Tryptic Soy Agar)	TSB[1]; 15 g agar [2], pH 7.3 ± 0.2 at 25 ºC
LB (Luria-Bertani Medium)	10 g peptone from casein[3], 5 g yeast extract[3],10 g sodium chloride[4], pH 7.0
MHB[1] (Mueller-Hinton Broth)	21.0 g of MHB (300 g of dehydrated infusion from beef, 17.5 g casein hydrolysate, 1.5 g starch, 7.3 ± 0.1 at 25ºC)
MHA[1] (Mueller-Hinton agar)	38.0 g of MHA (300 g of dehydrated infusion from beef, 17.5 g casein hydrolysate, 1.5 g starch, 17 g agar, 7.3 ± 0.1 at 25ºC)

[1] Oxoid Ltd, Basingstoke, Hampshire, England; [2] Scharlau Chemie SA, Barcelona, Spain; [3] Merck, Darmstadt, Germany; [4] Panreac Química, Barcelona, Spain.

1.6 Cell culture media.

McCoy's 5A medium (Lonza BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich Química SA, Madrid, Spain), L-glutamine (2mM final

concentration, Lonza BioWhittaker, Verviers, Belgium), 0.5 mL of fluconazole (Diflucan® 2mg/mL infusion, Pfizer, Amboise, France) and 5 mL of Penicillin-Streptomycin Solution Stabilized (cat.no. P4333, Sigma-Aldrich Chemie GmbH, Stenheim, Germany) for 500 mL McCoy's 5A.

2 - Methods:

2.1 Determination of minimum inhibitory concentrations (MIC) of bacteria

MICs of the 30 hydantoin derivatives (**SZ-2, SZ-7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, P10, P11, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, KK-XV, Thioam-1, JHF-1, JHC-2, JHP-1, Fur-2, GL-1, GL-7, GL-14, GL-16**, **GL-18)** against *Salmonella* Enteritidis NCTC 13349, wild-type *Escherichia coli* K-12 AG100 (*argE3 thi-1 rpsL xyl mtl* delta (*gal-uvrB*) *supE44*), *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were assessed by the broth microdilution method, in 96-well microplates, according to the CLSI guidelines (CLSI, 2006). The bacterial strains were incubated overnight, at 37°C in 5 ml of Mueller Hinton broth (MHB). The inoculums used for MIC determination were prepared by adjusting the optical density of the culture to the 0.5 McFarland standard. The bacterial suspension was diluted to 10^{-3} in Mueller Hinton broth (10 µL of inoculum in 10 mL of MHB). Aliquots of 100 µL of the diluted inoculum were then dispensed to each well of the microplate that contained serial two-fold dilutions of the compounds, starting from 500 µg/mL of final concentration. The final volume was 200 µL in each well. The microplates were then incubated at 37 ºC and the results registered after 16 and 18 hours of incubation. In order to assure that the hydantoin derivatives did not affect the cellular viability, the concentrations used did not exceed ½ of the MIC. The MIC was defined as the lowest concentration of the agent that produced no detectable evidence of growth (turbidity). The MIC for each agent and bacterium was determined three times.

2.2 Assay for antiproliferative and cytotoxic effect on mouse lymphoma cells

The effects of increasing concentrations of the hydantoin derivatives on cell growth were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in a volume of 100 µL in McCoy's 5A medium. Then, $6x10^3$ (for antiproliferative effect) or $2x10^4$ cells (for cytotoxicity assay) in 50 µL of medium, respectively, were added to each well, with the exception of the

medium control wells. The culture plates were further incubated at 37°C for 72 h (antiproliferative effect assay) and for 24 h (cytotoxicity assay); at the end of the incubation period, 15 µL of MTT (thiazolyl blue tetrazolium bromide, Sigma, St. Louis, MO, USA) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37°C for 4 h, 100 µL of 10 % sodium dodecyl sulfate (SDS, Sigma) in 0.01M HCl was measured into each well and the plates were further incubated at 37°C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of the cell growth was determined according to the formula:

$$ID_{50} = \quad 100 - \left[\frac{OD\,sample - OD\,medium\,control}{OD\,cell\,control - OD\,medium\,control} \right] x100$$

Where ID_{50} is defined as the inhibitory dose that reduces the growth of the compound- exposed cells by 50%.

2.3 Real-time semi-automated fluorometric method: accumulation assay to monitor efflux pump activity in bacteria

EB is widely used to quantify the transport across the envelope of the bacterial cell, because it generates a quantifiable signal. This signal is low outside and amplified inside the cell (VIVEIROS *et al,* 2010).

To screen the efflux modulating effect of the thirty hydantoin derivatives in the four strains of bacteria, a semi-automated method was used that demonstrates and quantifies the accumulation of EB on a real-time basis, developed by Viveiros *et al* (VIVEIROS et al, 2008). This method was carried out using the real-time thermocycler Rotor-Gene™ 3000 (Corbett Research, Sydney, Australia). The instrument allows the selection of the excitation and emission wavelengths that for EB are 530 nm band-pass and 585 nm high-pass filters, respectively. The fluorescence was acquired in cycles of 60 seconds, at 37 ºC.

Bacteria were grown in the respective media until they reached the optical density (OD) of 0.6 (1 x 10^8 CFU/mL, where CFU is colony forming unit), at 600 nm, which is their mid log phase. *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were grown in TSB medium, in addition *E. coli* AG 100 and *Salmonella* Enteritidis NCTC 13349 were grown in MHB. In order to remove the medium, the cultures were centrifuged for 3 minutes at 13000 rpm and washed twice in PBS. The final OD_{600} was adjusted to 0.6. Aliquots of 48 µL were transferred to 0.2 mL microtubes. Different concentrations of EB were added to PBS and used in aliquots of 48 µL: for *Staphylococcus aureus* ATCC 25923 the final concentration of EB was 0.25 µg/mL (COUTO *et al,* 2008), for *Enterococcus faecalis* ATCC 29212 the final concentration was 0.5 µg/mL (SPENGLER *et al*, 2009a), for *E. coli* AG 100 the final concentration was 1 µg/mL (VIVEIROS *et al,* 2009) and for *Salmonella* Enteritidis NCTC 13349 the final concentration was 1 µg/mL (AMARAL *et al,* 2010). Then 5 µL of the stock solutions of the compounds were added to the cells at three different concentrations (40, 80 and 120 µg/mL), individually. The tubes were then placed into the RotorGene 3000™ thermocycler (Corbett Research, Sydney, Australia), which provides, on a real-time basis, the assessment of transport kinetics of EB accumulation. The data obtained were analysed by Rotor-Gene Analysis Software 6.1 (Build 93) by Corbett Research (Sydney, Australia). Each accumulation assay was performed three distinct times.

From the real-time data, the relative final fluorescence (RFF) of the last time point (minute 60) of the EB accumulation assay was calculated according to the formula:

$$\text{Activity of the compound (RFF)} = \frac{\mathbf{RF}\ \text{treated} - \mathbf{RF}\ \text{untreated}}{\mathbf{RF}\ \text{untreated}}$$

The relative final fluorescence (RFF) value is the difference between the relative fluorescence (RF) at the last time point of the EB retention curve of cells in the presence of the inhibitor and the RF at

the last time point of the EB retention curve of the untreated solvent control, divided by the untreated solvent control. The solvent used in the assays was DMSO.

The mean of RFF of the three independent assays was calculated for each compound at different concentrations (40, 80 and 120 μg/mL).

2.4 Real-time semi-automated fluorometric method: accumulation assay to monitor ABCB1 (P-glycoprotein) pump activity in mouse lymphoma cells

The cells were adjusted to a density of 2 x 6^{10} cells/mL, centrifuged at 2000 x g for 2 minutes and resuspended in PBS. The cell suspension was distributed in 90 μL aliquots into 0.2 mL tubes. Volumes of 5 μL of the tested compounds stock solutions were added at different concentrations (4 and 40 μg/mL) individually, and the samples incubated for 10 minutes at room temperature. After this incubation, 5 μL (1 μg/mL final concentration) of EB were then added to the samples and the tubes were placed in the Rotor-Gene™ 3000 and the fluorescence monitored on a real-time basis. Prior to the assay, the instrument was programmed for temperature (37 ºC), the appropriate excitation and emission wavelengths of EB (530 nm of band-pass and 585 nm high-pass, respectively). The fluorescence was acquired in cycles of 60 seconds. The data obtained were analysed by Rotor-Gene Analysis Software 6.1 (Build 93) by Corbett Research (Sydney, Australia). Each accumulation assay was performed three distinct times. The RFF values were calculated according to the formula:

$$\text{Activity of the compound (RFF)} = \frac{\mathbf{RF}\ \text{treated} - \mathbf{RF}\ \text{untreated}}{\mathbf{RF}\ \text{untreated}}$$

The relative final fluorescence (RFF) value is the difference between the relative fluorescence (RF) at the last time point of the EB retention curve of cells in the presence of the inhibitor and the RF at

the last time point of the EB retention curve of the untreated solvent control, divided by the untreated solvent control. The solvent used in the assays was DMSO.

The mean of the three independent experiments was calculated for each compound at two concentrations (4 and 40 μg/mL).

IV - RESULTS

1 - Multidrug resistance in bacteria

One of the main mechanisms of antibacterial resistance is the prevention of antimicrobials from reaching their intended targets in bacterial cells. Decreased permeability, due to the lipopolysaccharides present in the outer membrane, or decrease of porins (restricted to Gram-negatives), and the increasing efflux of drugs by overexpressed efflux pumps, contribute to the emergence of MDR phenotypes in clinical isolates. In contrast to the decreased permeability that prevents the entrance of drugs into bacteria, EPs extrude a wide range of noxious agents, such as toxins or antimicrobials (VIVEIROS *et al,* 2010).

Fluorometry has been used to detect and quantify the transport of fluorescent substrates, such as EB, across the cell wall of bacteria. The use of fluorescent substrates, such as rhodamine 123, was the basis upon which flow cytometry became a worldwide recognized technique (VIVEIROS *et al,* 2010; DAVEY & KELL, 1996).

EB has been demonstrated to be appropriate as a probe for these experiments because emits weak fluorescence outside the cells (aqueous solution) and becomes strongly fluorescent inside the cells, as this is a nonpolar and hydrophobic environment (VIVEIROS *et al,* 2010).

To screen the efflux pump modulating effect of the thirty hydantoin derivatives, a semi-automated method was used, developed by Viveiros *et al* (VIVEIROS *et al,* 2008). The aim of this method is to easily and accurately detect and quantify the transport of EB through the bacterial cell wall, at working concentrations that do not affect cell viability or the cellular function. This method allows the real-time assessment of the balance between accumulation of EB through passive diffusion and extrusion via efflux of the substrate, allowing the monitoring of the efflux of substrates that are accumulated by a large population of cells.

The semi-automated fluorometric method can be employed to compare intrinsic and over-expressed efflux systems of bacteria, presenting advantages comparing to the flow cytometry. It is user-friendly, provides real-time assessment of the activity of the modulators and uses an inexpensive substrate, i.e. EB. However, the semi-automated fluorometric method is less sensitive than the flow cytometry (VIVEIROS *et al,* 2010; BREHM-STECHER & JOHNSON, 2004).

2 - Accumulation of EB by bacterial efflux systems

To screen the efflux pump modulating activity of the thirty hydantoin derivatives in bacteria, four well characterized reference strains were used. Two Gram-positive and two Gram-negative strains were tested: *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, *Salmonella* Enteritidis NCTC 13349 and *Escherichia coli* AG100, respectively.

The selection criterion was the different cell wall structure that is much more permeable in Gram-positives. Therefore, a semi-automated real-time fluorometric method was performed that demonstrates and quantifies the accumulation of EB on a real-time basis (VIVEIROS *et al,* 2008).

In order to test the thirty hydantoin derivatives, concentrations were chosen that did not exceed ½ of the MIC of the compounds against *Salmonella* Enteritidis NCTC 13349, *Escherichia coli* AG100, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. Thus, three different concentrations were applied in fluorometry such as 40, 80 and 120 μg/mL.

MIC is considered the "gold standard" method for the determination of the susceptibility of microorganisms to antimicrobials. It is defined as the lowest concentration at which an antimicrobial inhibits the visible growth of a microorganism after overnight incubation, it means that the growth of the microorganisms has to be read after the 16^{th} and 18^{th} hour (CLSI, 2006).

The MICs of EB and the thirty hydantoin derivatives were determined by broth microdilution method to ensure that the concentrations of each agent employed for assessment of their ability to

affect accumulation of EB would be well below that which affects the replication of the strains studied, furthermore the concentrations of the efflux pump modulator hydantoins would not affect the viability of the cells.

The exact MIC could not be determined because of the precipitation in Mueller-Hinton medium showed by **SZ-2**, **SZ-7**, **BS-1**, **JH-63**, **MN-3**, **P7**, **P10**, **AD-26**, **RW-13**, **AD-29**, **KF-2**, **Thioam-1** and **JHC-2** at high concentrations, furthermore the amount of compounds available was limited. The MICs of the compounds were above 500 mg/L for all the strains studied.

The relative final fluorescence (RFF) value permits the comparison of the inhibitory effect of hydantoin derivatives at the last time point, i.e. minute 60. It is the difference between the relative fluorescence (RF) at the last time point (minute 60) of the EB retention curve of cells in the presence of the inhibitor and the RF at the last time point of the EB retention curve of the untreated solvent control, divided by the RF of the solvent control. The RF values were obtained from the real-time data.

Compounds that showed RFF values above zero were considered effective in the inhibition or modification of efflux pump activity. Compounds that presented a RFF value equal to zero had no effect on the EB accumulation, furthermore, hydantoin derivatives that demonstrated RFF values under zero were considered not effective because of the possible interference with other cell functions or increase in efflux. In order to select the best compounds, hydantoin derivatives that demonstrated RFF values over 0.3 were considered the most effective ones.

In this work, reference strains were used, as models of the intrinsic efflux pump systems of bacteria. The RFF values of the accumulation of EB were calculated and compared in the presence of the hydantoin derivatives. The EB concentrations used in the present study differed based on previous studies. The EB concentrations applied were as follows: *Salmonella* Enteritidis NCTC 13349 1 µg/mL (AMARAL *et al,* 2010), *E. coli* AG 100 1 µg/mL (VIVEIROS *et al,* 2008), *Staphylococcus*

aureus ATCC 25923 0. 25 μg/mL (COUTO *et al,* 2008) and *Enterococcus faecalis* 29212 0.5 μg/mL) (SPENGLER *et al,* 2009a).

The thirty derivatives have also been tested in order to detect their auto-fluorescence and interaction with ethidium bromide. The compounds were applied at the highest concentration (120 μg/mL) in the presence and absence of EB in PBS. From the thirty hydantoin derivatives studied, **SZ-7** interacted with EB, presenting fluorescence intensity of 30 arbitrary units.

Since the semi-automated fluorometric method analyses the balance between accumulation and efflux of EB, all the assays were performed in comparison to two different controls: one that contains the inoculum and EB, and the other with one containing the inoculum, EB and DMSO which is the solvent of the compounds.

2.1 Effect of hydantoins on Gram-negative bacteria

2.1.1 Accumulation of EB by S*almonella* Enteritidis NCTC 13349

Table 6. Relative final fluorescence (RFF) based on the accumulation of EB (1 µg/mL) by *Salmonella* Enteritidis NCTC 13349 in the presence of hydantoin derivatives.

	Relative Final Fluorescence (RFF) S*almonella* Enteritidis NCTC 13349		
Compounds	**40 µg/mL**	**80 µg/mL**	**120 µg/mL**
Thioam-1	1.1	0.8	1.0
SZ-2	0.7	2.1	2.8
P3	0.5	1.1	1.4
RW-15b	0.4	1.0	0.9
AD-26	0.4	0.8	0.4
GL-7	0.3	0.4	0.6
AD-29	0.3	0.5	0.3
GL-18	0.2	0.5	0.4
P7	0.2	0.3	0.3
Fur-2	0.2	-0.1	-0.1
GL-16	0.2	0	0
MN-3	0.1	0.2	0.4
JHC-2	0.1	0.2	0.3
Mor-1	0.1	0.2	0.1
JHF-1	0.1	0.1	0
JHP-1	0.1	-0.1	-0.1
GL-1	0.1	-0.1	-0.1
SZ-7	0	1	3.7
KK-XV	0	0	-0.1
KF-2	0	0	0.1
JH-63	0	0.3	0.5
BS-1	0	0.1	0.1
GG-5k	0	-0.2	-0.2
P10	-0.1	0.2	0.2
RW-13	-0.1	0	-0.1
PDPH-3	-0.1	0.1	-0.1
TD-7k	-0.1	-0.1	-0.1
GL-14	-0.1	-0.1	-0.2
LL-9	-0.1	0.1	-0.1
P11	-0.3	-0.1	0

The RFF values of the accumulation of EB (1 µg/mL) in the presence of hydantoin compounds on *Salmonella* Enteritidis NCTC 13349 are shown in Table 6.

As demonstrated by Table 6, the most effective compounds are the following: **Thioam-1**, **SZ-2**, **P3**, **RW-15b**, **SZ-7**, **AD-26**, **AD-29**, **GL-7**, **GL-18** and **P7**. These derivatives significantly increased the retention of EB inside the cells. Furthermore, **SZ-7** was the most potent inhibitor, as demonstrated by the ranking (Table 6), especially when used at 120 µg/mL.

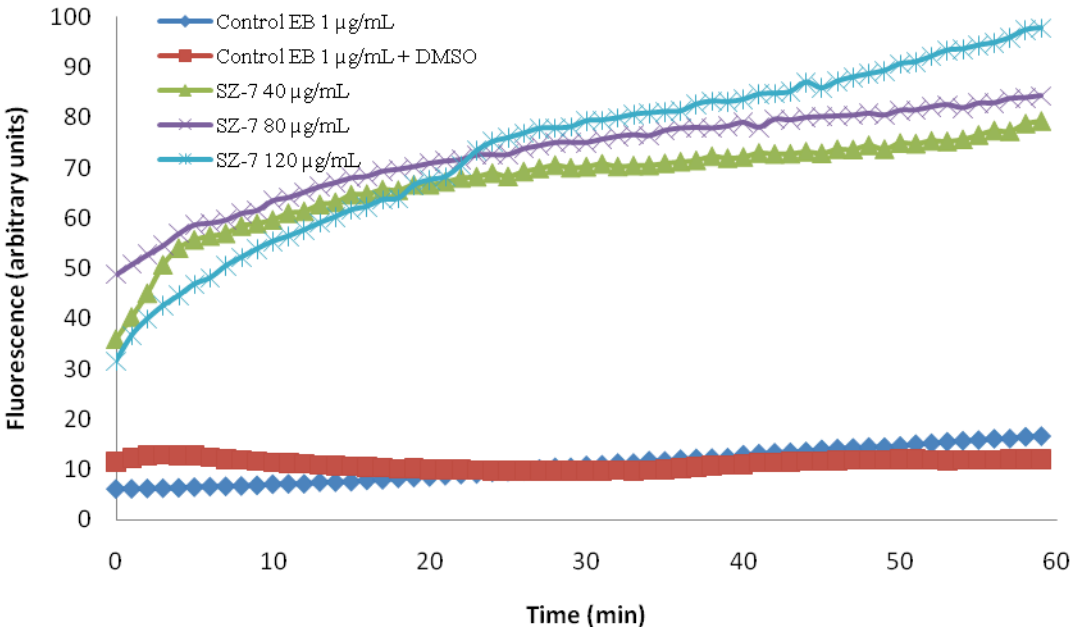

Figure 12. Accumulation of EB (1µg/mL) in the presence of SZ-7 (40, 80 and 120 µg/mL) on *Salmonella* Enteritidis NCTC 13349.

As evident from Figure 12, in the presence of **SZ-7**, the accumulation of EB was increased, as compared to the controls, where the modulator was not present. The effect of **SZ-7** on EB accumulation was dose-dependent, namely the increasing accumulation of EB is proportional to the increasing concentrations of the compound.

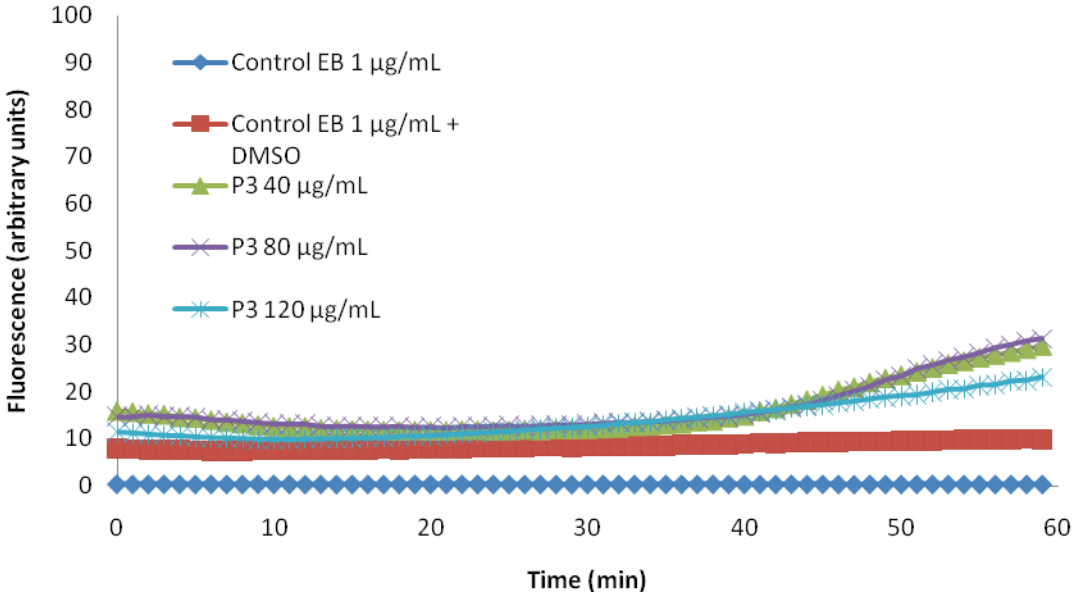

Figure 13. Accumulation of EB (1µg/mL) in the presence of P3 (40, 80 and 120 µg/mL) on *Salmonella* Enteritidis NCTC 13349.

As revealed by Figures 13 and 14, **P3** and **SZ-2** possessed good efflux modulating effect, respectively. Among the derivatives studied, **Thioam-1** and **RW-15b** increased significantly the accumulation of EB. In addition, **AD-26**, **GL-7**, **AD-29**, **GL-18** and **P7** were also active and can be good candidates for further studies.

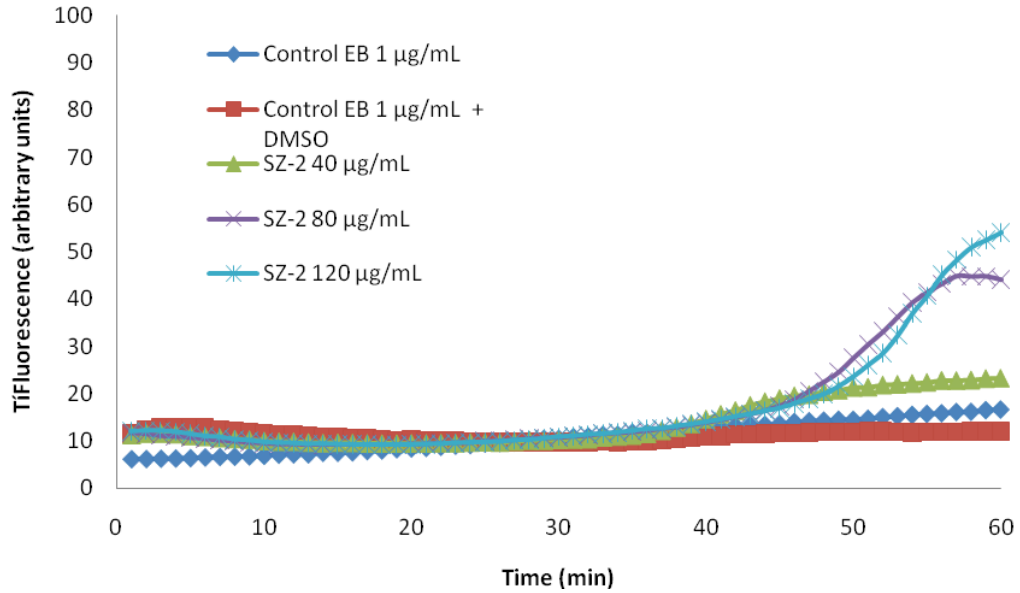

Figure 14. Accumulation of EB (1µg/mL) in the presence of SZ-2 (40, 80 and 120 µg/mL) on *S.* Enteritidis NCTC 1334.

2.1.2 Accumulation of EB by *Escherichia coli* AG 100

Table 7. Relative final fluorescence (RFF) based on the accumulation of EB (1 µg/mL) by *E. coli* AG100 in the presence of hydantoin derivatives.

Relative Final Fluorescence (RFF) *E. coli* AG100			
Compounds	**40 µg/mL**	**80 µg/mL**	**120 µg/mL**
AD-29	1.7	1.6	1.2
RW-15b	1.5	1.1	1.3
AD-26	1.1	1.0	1.2
Thioam-1	1.0	0.5	0.4
GL-18	0.6	0.7	0.8
P3	0.5	1.0	1.3
KF-2	0.4	0.5	0.4
SZ-2	0.3	0.6	0.7
RW-13	0.3	0.3	0.3
BS-1	0.3	0.2	0.2
GL-16	0.3	0.2	0
JH-63	0.2	1.2	1.3
P10	0.2	0.2	0.3
PDPH-3	0.2	0.2	0.3
Mor-1	0.2	0.2	0.3
KK-XV	0.2	0.1	0
MN-3	0.1	0.9	1.0
P7	0.1	0.2	0.4
JHC-2	0.1	0.2	0.3
GL-7	0.1	0.2	0.3
P11	0.1	0.2	0.2
LL-9	0	0.8	0.5
JHP-1	0	0	-0.1
GL-1	0	0	0
JHF-1	0	0	-0.1
Fur-2	0	0	0.1
GL-14	0	-0.1	-0.1
TD-7k	0	0	0
GG-5k	0	0	0
SZ-7	-2.4	-1.6	-0.3

Table 7 presents the RFF values of the accumulation of EB (1 µg/mL) in the presence of hydantoin derivatives on *E. coli* AG100.

As demonstrated in Table 7, almost all of the compounds were effective in efflux pump modulation. In addition, the most effective compounds were **AD-29**, **RW-15b**, **AD-26**, **Thioam-1**, **GL-18**, **P3**, **KF-2** and **RW-13**. According to Table 7, **SZ-2**, **JH-63** and **MN-3** had also remarkable activity.

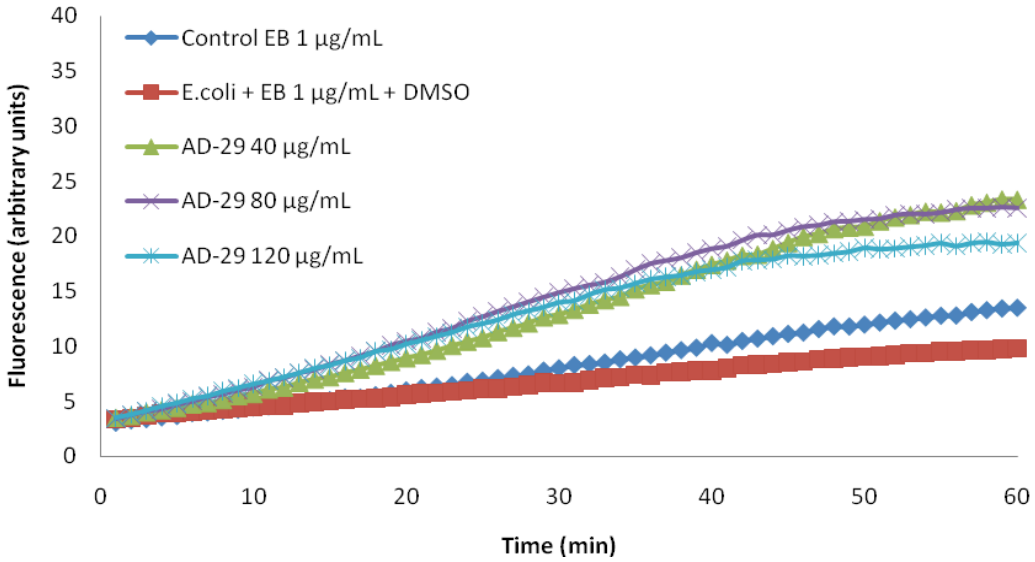

Figure 15. Accumulation of EB (1μg/mL) by *E. coli* AG 100 in the presence of AD-29 (40, 80 and 120 μg/mL).

As demonstrated in Figure 15, **AD-29** effectively inhibited the efflux of EB, however it was more effective at low concentration. Compound **Thioam-1** showed similar characteristics, because it was more effective at 40 μg/mL than at higher concentrations (80 and 120 μg/mL).

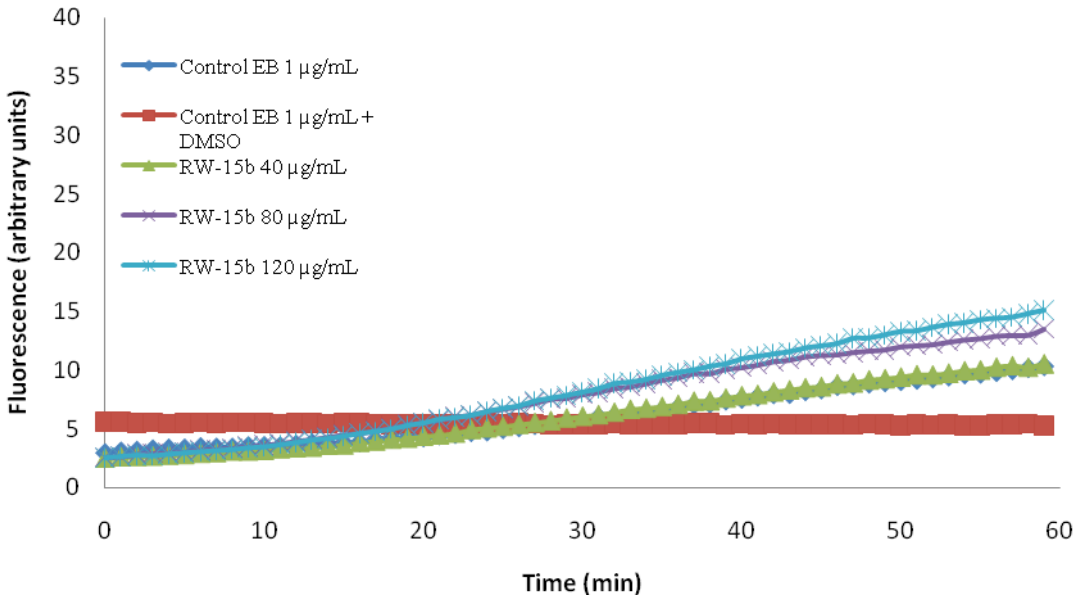

Figure 16. Accumulation of EB (1 µg/mL) by *E. coli* AG 100 in the presence of RW-15b (40, 80 and 120 µg/mL).

As shown by Figure 16, **RW-15b**, that is one of the compounds with higher ranking, caused EB accumulation, demonstrating thus modulating effect

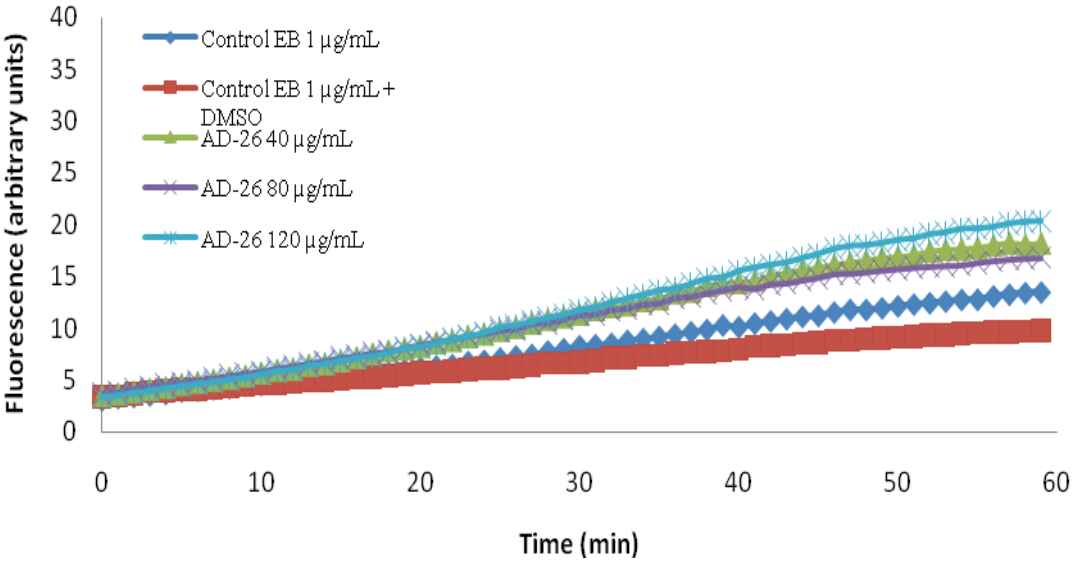

Figure 17. Accumulation of EB (1µg/mL) in the presence of AD-26 (40, 80 and 120 µg/mL) on *Escherichia coli* AG100.

As evident from Figure 17, **AD-26** effectively inhibited the extrusion of EB compared to the controls, moreover the RFF values were similar for all the three concentrations applied, indicating that the compound might be active at lower concentrations.

2.2. Effect of hydantoins on Gram-positive bacteria

2.2.1 Accumulation of EB by *Staphylococcus aureus* ATCC 25923

Table 8. Relative final fluorescence (RFF) based on the accumulation of EB (0.25 µg/mL) by *Staphylococcus aureus* ATCC 25923 *in* the presence of hydantoin derivatives.

Relative Final Fluorescence (RFF) *Staphylococcus aureus* ATCC 25923			
Compounds	**40 µg/mL**	**80 µg/mL**	**120 µg/mL**
GL-18	0.5	0.7	0.8
MN-3	0.4	0.2	0.2
GL-16	0.3	0.3	0.3
TD-7K	0.2	0	0.1
Sz-2	0.2	-0.1	-0.1
GG-5K	0.2	-0.1	-0.2
GL-14	0.1	0	0.1
AD-26	0.1	-0.1	-0.2
Sz-7	0.1	-0.3	0.3
GL-7	0	0	0.1
P10	0	-0.3	-0.1
P11	0	-0.1	-0.1
JHC-2	0	0	0
JH-63	0	-0.2	-0.2
KF-2	-0.1	-0.1	0
BS-1	-0.1	-0.1	-0.1
P3	-0.1	-0.1	-0.2
Mor-1	-0.1	-0.3	-0.1
LL-9	-0.1	-0.3	-0.2
AD-29	-0.1	-0.4	-0.2
GL-1	-0.2	-0.1	0.1
Fur-2	-0.2	-0.2	-0.1
PDPH-3	-0.2	-0.2	-0.2
JHF-1	-0.3	0	-0.1
JHP-1	-0.3	-0.1	-0.2
KK-XV	-0.3	-0.1	-0.2
P7	-0.3	-0.1	-0.3
RW-15b	-0.3	-0.3	-0.3
RW-13	-0.3	-0.4	-0.3
Thioam-1	-0.3	-0.7	-0.8

The ranking of compounds based on RFF values of the accumulation of EB (0.25 µg/mL) in the presence of the thirty hydantoin derivatives is demonstrated in Table 8. As shown by Table 8, the majority of the hydantoin compounds were not effective on *Staphylococcus aureus* ATCC 25923.

GL-18 (shown by Figure 18) was the most potent inhibitor, furthermore **GL-16** and **MN-3** could modify the efflux activity of the strain, promoting the retention of EB inside the cells.

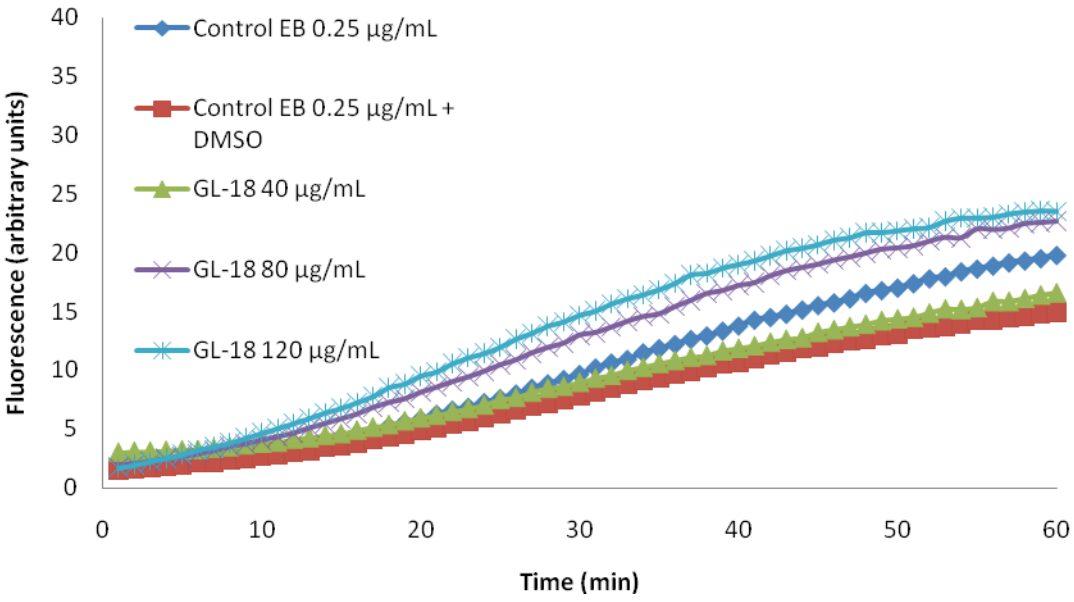

Figure 18. Accumulation of EB (0.25 µg/mL) in the presence of SZ-7 (40, 80 and 120 µg/mL) on *Staphylococcus aureus* ATCC 25923.

2.2.2 Accumulation of EB by *Enterococcus faecalis* ATCC 29212

Table 9. Relative final fluorescence (RFF) based on the accumulation of EB (0.5 µg/mL) by *Enteroccocus faecalis* ATCC 29212 in the presence of hydantoin derivatives.

Relative Final Fluorescence (RFF) *Enterococcus faecalis* ATCC 29212			
Compounds	**40 µg/mL**	**80 µg/mL**	**120 µg/mL**
GL-14	0.3	0.8	1.1
LL-9	0.3	0.1	-0.1
P3	0.2	0.4	0.3
JHF-1	0.2	0.2	0.1
SZ-2	0.2	0	0.1
BS-1	0.2	0	0.1
MN-3	0.1	0.1	0.1
JHP-1	0.1	0.2	0.2
JHC-2	0.1	0.1	0.2
Fur-2	0.1	0.1	0.1
GL-1	0.1	0.1	0
GL-7	0	0.2	0.5
P7	0	0.2	0
GL-16	0	0	0.2
GG-5k	0	0	0
KK-XV	0	0	0
JH-63	0	-0.2	-0.1
Mor-1	-0.1	0.1	0.1
PDPH-3	-0.1	0	0.1
KF-2	-0.1	0.1	-0.1
P10	-0.1	0	0
TD-7k	-0.1	0	0
AD-29	-0.1	0	-0.1
AD-26	-0.1	-0.1	-0.1
Thioam-1	-0.1	-0.3	-0.5
RW-15b	-0.2	-0.3	-0.3
GL-18	-0.2	-0.1	0.2
P11	-0.2	-0.2	-0.2
RW-13	-0.3	-0.2	-0.2
SZ-7	-1.2	-0.7	0.1

Table 9 demonstrates the RFF values of the accumulation of EB (0.5 µg/mL) in the presence of the hydantoin derivatives on *Enterococcus faecalis* ATCC 29212.

As shown by Table 9, **GL-14** and **GL-7** inhibited the extrusion of EB in a dose dependent manner. Compound **P3** showed slight efflux modulating effect.

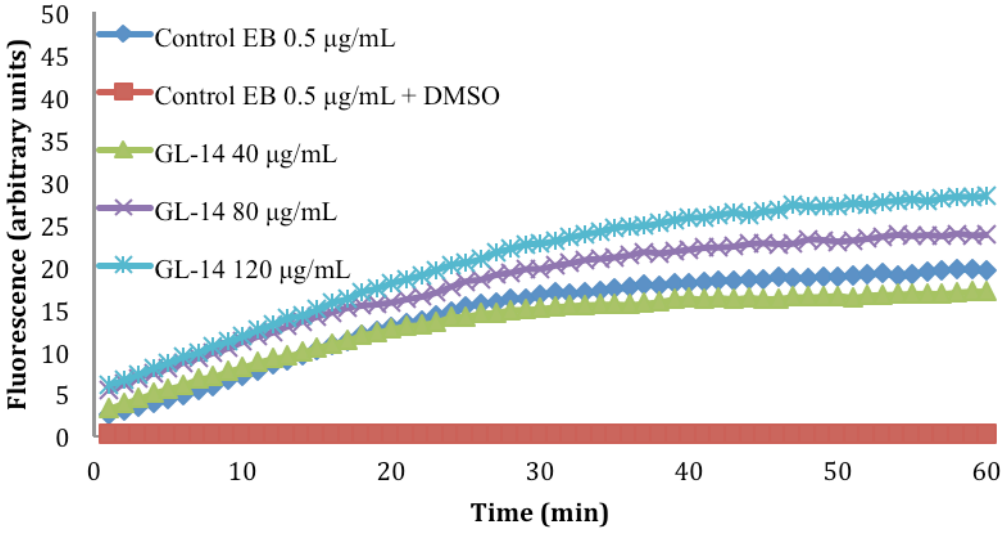

Figure 19. Accumulation of EB (0.5 µg/mL) in the presence of GL-14 (40, 80 and 120 µg/mL) on *Enterococcus faecalis* ATCC 29212.

As indicated in Figure 19, compound **GL-14** modified the retention of EB, furthermore **GL-14** was more active compared to **GL-7**, indicating the importance and the position of the –COOH group.

3 - Efflux pump modulating activity of hydantoin derivatives on cancer cells

Resistance to chemotherapy is a big concern in the treatment of cancer. As chemotherapy is the treatment of choice for ~50% of all types of cancer, acquired resistance to chemotherapy must be studied (AMBUDKAR *et al,* 2005).

Overexpression of drug transporters such as ABCB1 (P-gp) is the main mechanism by which cancer cells avoid the toxic effects of chemotherapeutic drugs. The clinical importance of ABCB1 derives from the fact that over-expression of P-gp is often associated with multidrug resistance.

In order to reduce the ABCB1 related consequences in the failure of chemotherapy, the development of potent but nontoxic inhibitors is fundamental. Because of the importance of ABCB1, the screening of ABCB1 modulating agents has to be incorporated into the drug discovery process.

Flow cytometry is the standard method to screen P-gp modulators (ARCECI, 1993). In this method the substrate used is rhodamine 123. Therefore, the aim of this assay is to find P-gp modulators that inhibit the extrusion of rhodamine 123. The method is very sensitive but does not provide a real-time assessment of the modulator, uses a very expensive instrument and needs a well experienced technician. For this purpose, Spengler *et al* developed a new fluorometric method (SPENGLER *et al,* 2009b) based on the previously described semi-automated method used to monitor bacterial efflux systems (VIVEIROS *et al,* 2008).

The fluorometric method (SPENGLER *et al,* 2009b) has been applied to assess the activity of ABCB1 in multi-drug resistance (MDR) T-lymphoma cells transfected with the human ABCB1 gene, which overexpress the ABCB1 transporter, and in parental (PAR) T-lymphoma cell line, showing normal ABCB1 expression level. The fluorometric method allows the comparison of

ABCB1 modulators and screen their effect in cells with normal and overexpressed ABCB1 efflux activity. In contrast to the flow cytometry, this new method uses etidium bromide (EB) instead of rhodamine 123. EB is a common substrate of bacterial efflux transporters and P-glycoprotein and an inexpensive fluorochrome. The semi-automated fluorometric method assesses the EB accumulation in various cells and in a real-time manner, contrasting to the flow cytometry that assesses rhodamine 123 accumulation at one point of time in the cell population. However, flow cytometry is much more sensitive and presents different cell populations in contrast to the fluorometric method that shows the bulk signal of the population.

3.1 Antiproliferative and cytotoxic effects of hydantoin derivatives on L5178 (parental, PAR) mouse T-cell lymphoma cells and its human *ABCB1* (*MDR1)*-transfected subline (MDR)

MTT assay to assess cell proliferation and cytotoxicity

The MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is based on the metabolic activity of the cells and is widely used to quantify cell proliferation and cytotoxicity.

The principle of the MTT assay is that the tetrazolium ring in MTT is cleaved by dehydrogenases present in active mithocondria of the metabolically active cells, producing an insoluble MTT formazan product. Formazan is then solubilized with DMSO or 10% SDS and easily quantified by colorimetry.

Table 10. Antiproliferative and cytotoxic effects of hydantoin derivatives on L5178 (parental, PAR) mouse T-cell lymphoma cells and its human *ABCB1* (*MDR1)*-transfected subline (MDR).

	IC_{50} (µg/mL)			
Hydantoin derivatives	**MDR Antiproliferative effect**	**MDR Cytotoxic effect**	**PAR Antiproliferative effect**	**PAR Cytotoxic effect**
SZ-2[a*]	**25.61**	**48.33**	**29.40**	**67.12**
SZ-7[b*]	**4.54**	**3.84**	**4.60**	**4.41**
LL-9	54.19	54.12	64.49	54.19
BS-1[c*]	**15.21**	**60.07**	**8.6**	**16.42**
JH-63[d*]	**15.24**	**28.09**	**14.99**	**27.62**
MN-3[e*]	**10.55**	**16.97**	**8.24**	**13.89**
TD-7k	107.73	237.94	141.09	198.47
GG-5k	72.66	151.23	99.88	203.77
P3	**19.33**	**28.09**	**12.6**	**12.38**
P7[f*]	**87.13**	**18.15**	**30.85**	**43.77**
P10[g*]	112.09	286.50	56.31	89.65
P11	118.77	115.71	101.05	190.52
RW-15b	**53.42**	**37.97**	**31.17**	**44.48**
AD-26[h*]	**33.19**	**31.33**	**10.32**	**13.24**
RW-13[i*]	**11.95**	**19.12**	**5.31**	**14.83**
AD-29[j*]	**39.42**	**19.48**	**11.38**	**6.43**
KF-2	71.99	82.31	54.34	70.08
PDPH-3	90.10	99.36	23.84	16.00
Mor-1	76.80	240.24	34.92	120.22
KK-XV	78.67	245.56	84.95	171.27
Thioam-1[k*]	**5.272**	**3.92**	**4.65**	**6.85**
JHF-1	111.59	225.71	94.14	192.88
JHC-2	84.78	143.80	53.52	135.80
JHP-1**	**N.D.**	**N.D.**	**N.D.**	**N.D.**
Fur-2	94.64	426.01	91.01	283.76
GL-1	92.48	187.74	94.21	296.11
GL-7	47.34	313.42	53.36	174.09
GL-14	78.78	276.72	88.37	212.73
GL-16	100.99	208.35	102.42	206.16
GL-18	86.69	443.95	82.02	295.34
DMSO	1.09 %	2.39 %	1.04 %	6.17%

*precipitation, crystal-formation: [a] amorphous crystals, [b] amorphous crystals, [c] needle-like crystals,[d] amorphous crystals, [e] amorphous crystals, [f] oval crystals, [g] pine-leaf-like crystals, [h] amorphous crystals, [i] amorphous crystals, [j] needle-like crystals, [k] amorphous crystals

**complex formation with MTT in McCoy's 5A, IC_{50} non-determinable.

The compounds were tested for antiproliferative and cytotoxic effect against L5178 mouse T-cell lymphoma cells (parental, PAR) and its human *ABCB1 (MDR1)*-transfected subline (MDR).

As demonstrated in Table 10, from the listed compounds, **SZ-7** and **Thioam-1** showed the strongest antiproliferative and cytotoxic effect on PAR and MDR cells as well. The concentrations required to inhibit 50% of cell growth are shown in Table 10.

As demonstrated by Figure 20 and depicted by Table 10, some of the compounds showed precipitation in McCoy's 5A medium.

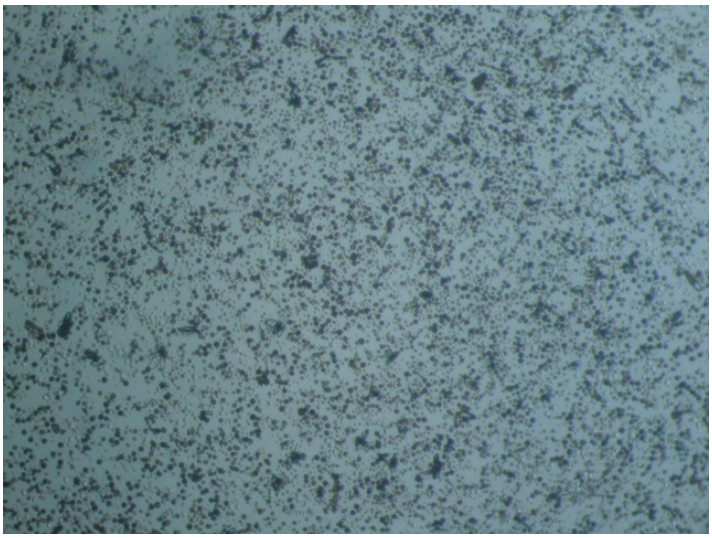

Figure 20. Precipitation shown by PAR cells in McCoy's 5A medium in the presence of AD-26 (200 µg/mL)

3.2 Accumulation assay of EB in efflux systems of cancer cells

The optimum concentration of EB for studying real-time accumulation on L5178 (parental, PAR) mouse T-cell lymphoma cells and its human *ABCB1* (*MDR1)*-transfected subline (MDR) is 1 µg/mL. This concentration permits the study of EB accumulation in the presence of EPIs without affecting the cell viability (SPENGLER *et al*, 2009b).

The semi-automated fluorometric method analyses the balance between accumulation and efflux of EB. All the assays were performed in comparison to two different controls: one that contains the untreated cells, PAR or MDR plus EB, and the other one containing PAR or MDR cells, EB and DMSO which is the solvent of the compounds.

The relative final fluorescence (RFF) value permits the comparison of the hydantoin derivatives on ABCB1 inhibition. The RFF is the difference between the relative fluorescence (RF) at the last time point (minute 60) of the EB retention curve of cells in the presence of the inhibitor and the RF at the last time point of the EB retention curve of the untreated solvent control, divided by the RF of the solvent control. The RFF values were obtained from the real-time data.

Compounds that showed RFF values above zero were considered effective in the inhibition or modification of ABCB1. Compounds that presented a RFF value equal to zero had no effect on the EB accumulation and hydantoin derivatives that demonstrated RFF values under zero were considered not effective because they increased the efflux activity or modified other cell functions. In order to select the best compounds, hydantoin derivatives that demonstrated RFF values over 0.3 were considered the most effective.

Table 11. Relative final fluorescence (RFF) based on the accumulation of EB (1 µg/mL) by PAR cells in the presence of hydantoin derivatives.

Relative Final Fluorescence (RFF) PAR cells		
Compounds	**4 µg/mL**	**40 µg/mL**
AD-29	0.6	0.4
KF-2	0.6	0
Thioam-1	0.6	-0.2
Fur-2	0.5	0.1
Mor-1	0.5	-0.1
MN-3	0.4	0.9
JHC-2	0.3	0
P3	0.2	0.4
BS-1	0.2	0.2
P7	0.2	0.2
P11	0.2	0.2
TD-7K	0.2	0
JH-63	0.1	0.9
SZ-7	0.1	0.6
JHF-1	0.1	0
GG-5K	0.1	-0.1
PDPH-3	0	0.7
SZ-2	0	0.2
GL-7	0	0.1
P10	0	0
AD-26	0	-0.1
GL-1	0	-0.1
RW-13	-0.1	0.1
GL-14	-0.1	0.1
LL-9	-0.1	0
JHP-1	-0.1	-0.1
GL-18	-0.2	0
RW-15b	-0.2	-0.1
KK-XV	-0.3	-0.2
GL-16	-0.3	-0.3

As demonstrated in Table 11, some of the hydantoin derivatives could modify the activity of ABCB1. The most effective ones were the following: **AD-29**, **KF-2**, **Thioam-1**, **Fur-2**, **Mor-1**, **MN-3**, **JHC-2**, **JH-63**, **SZ-7** and **PDPH-3**.

3.2.1 Effective compounds on PAR cells

As seen in Table 11, **AD-29**, **KF-2**, **Thioam-1**, **MN-3** and **JH-63** were the most effective compounds.

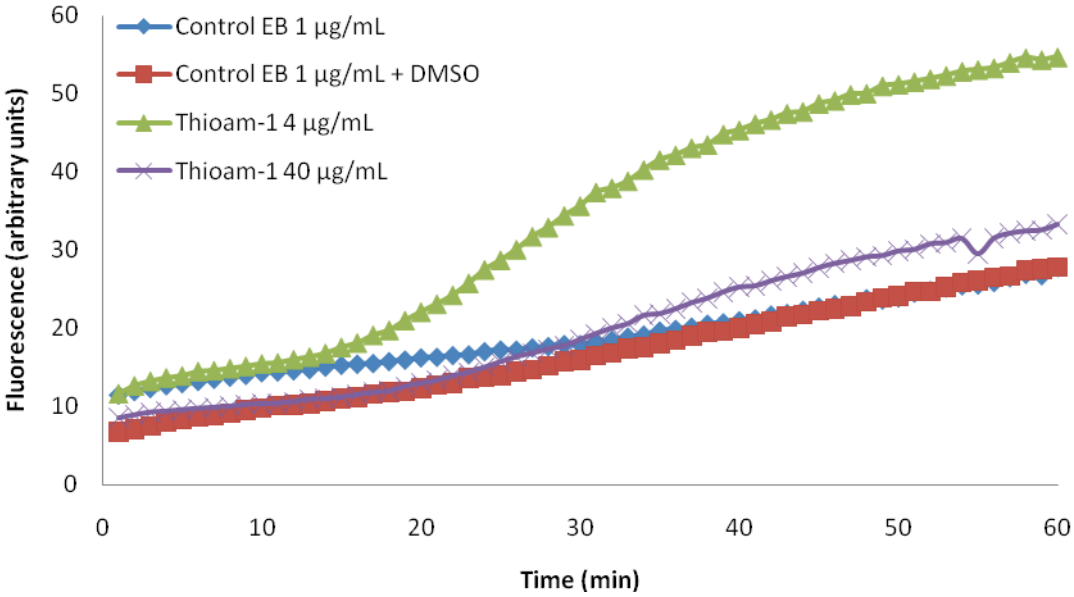

Figure 21. Accumulation of EB (1 µg/mL) in the presence of Thioam-1 (4 and 40 µg/mL) on PAR cells.

The activity of the hydantoin derivatives can be demonstrated by the example of two derivatives such as **Thioam-1** and **Fur-2**.

As presented in Figure 21, at low concentration (4 µg/mL), **Thioam-1** was more effective in contrast to the characteristics presented by this modulator at high concentration (40 µg/mL). Compound **Fur-2** showed the same activity, because it was more effective at low concentration than at 40 µg/mL (Figure 22).

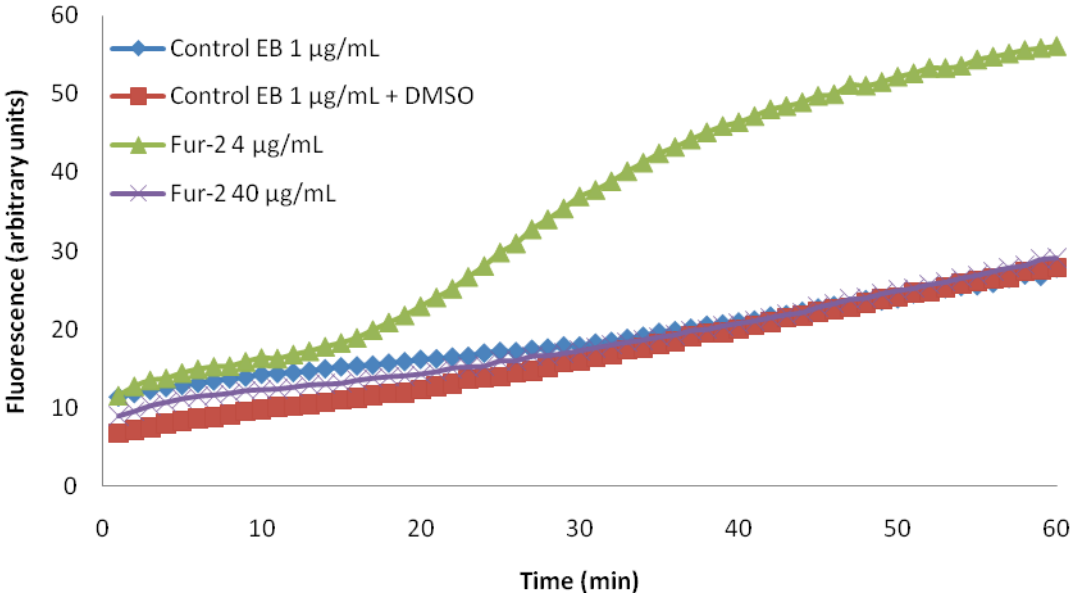

Figure 22. Accumulation of EB (1 µg/mL) in the presence of Fur-2 (4 and 40 µg/mL) on PAR cells.

Table 12. Relative final fluorescence (RFF) based on the accumulation of EB (1 µg/mL) by MDR cells in the presence of hydantoin derivatives.

Relative Final Fluorescence (RFF) MDR cells		
Compounds	**4 µg/mL**	**40 µg/mL**
GL-7	0.9	0
PDPH-3	0.8	0.8
KK-XV	0.8	0.3
AD-29	0.7	1.1
Thioam-1	0.7	0.1
SZ-7	0.6	1.6
KF-2	0.6	0.4
MN-3	0.5	0.7
RW-13	0.4	1.5
LL-9	0.4	0.3
P3	0.4	0.3
GL-1	0.3	0.2
TD-7K	0.3	-0.1
Mor-1	0.2	0
AD-26	0.1	0.4
GL-18	0.1	0.3
SZ-2	0.1	0.3
JHP-1	0.1	0.2
GL-14	0.1	0.2
JHF-1	0.1	0.1
GL-16	0.1	-0.2
JH-63	0	0.7
GG-5K	0	0.2
P10	0	0.1
JHC-2	0	-0.4
P11	-0.1	0
Fur-2	-0.1	0
P7	-0.2	-0.2
BS-1	-0.2	0.4
RW-15b	-0.3	1.3

As presented in Table 12, the majority of the hydantoin derivatives effectively inhibited the extrusion of EB in MDR cells, such as **PDPH-3**, **GL-7**, **KK-XV**, **AD-29**, **Thioam-1**, **SZ-7**, **KF-2**, **MN-3**, **RW-13**, **LL-9**, **P3**, **GL-1**, **TD-7K**, **AD-26**, **GL-18**, **SZ-2**, **JH-63**, **BS-1** and **RW-15b**.

3.2.2 Effective compounds on MDR cells

As presented in Table 12, **RW-13**, **RW-15b**, **AD-29** and **SZ-7** were the most effective compounds in MDR cells.

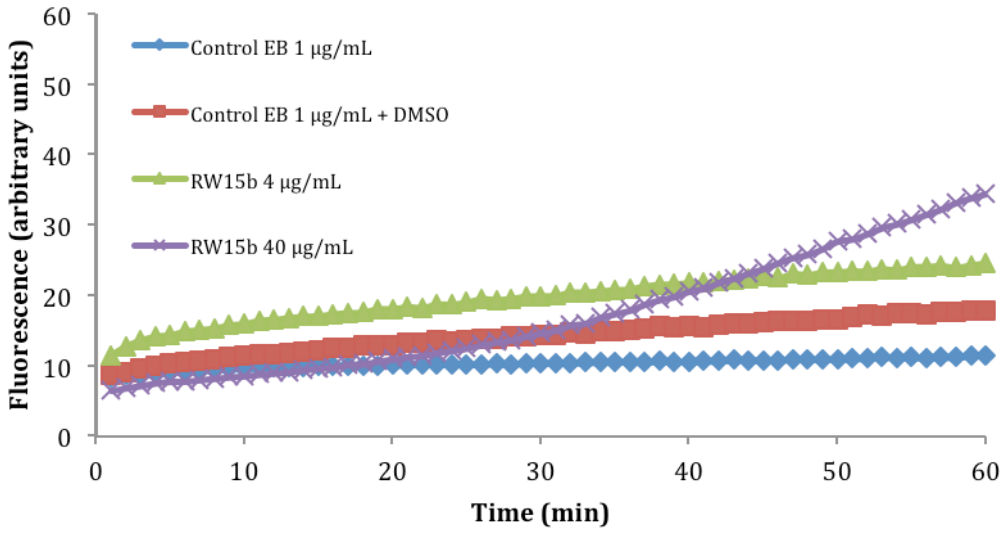

Figure 23. Accumulation of EB (1 µg/mL) in the presence of RW-15b (4 and 40 µg/mL) on MDR cells.

As demonstrated in Figure 23, **RW-15b** effectively inhibited the extrusion of EB by ABCB1 in comparison with the controls.

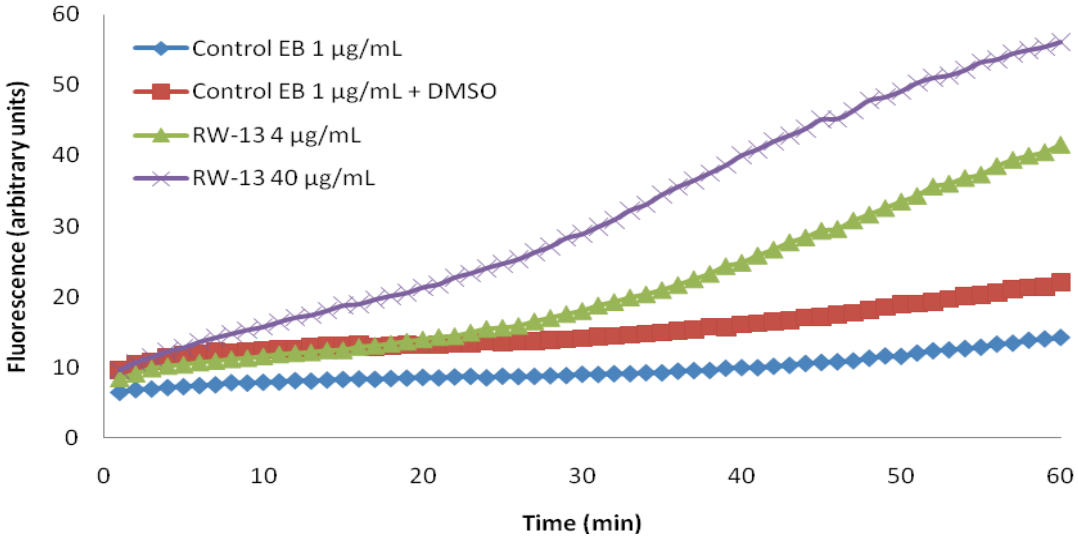

Figure 24. Accumulation of EB (1 µg/mL) in the presence of RW-13 (4 and 40 µg/mL) on MDR cells.

As evident from Figure 24, **RW-13** was very effective promoting the accumulation of EB, furthermore this derivative could inhibit the activity of ABCB1 in a dose-dependent manner.

3.2.3 Effective compounds on MDR and PAR cells

As shown by Figures 25, 26 and 27, there were some compounds effective on PAR and MDR cells as well: **PDPH-3**, **AD-29**, **Thioam-1**, **SZ-7**, **KF-2**, **MN-3** and **JH-63**.

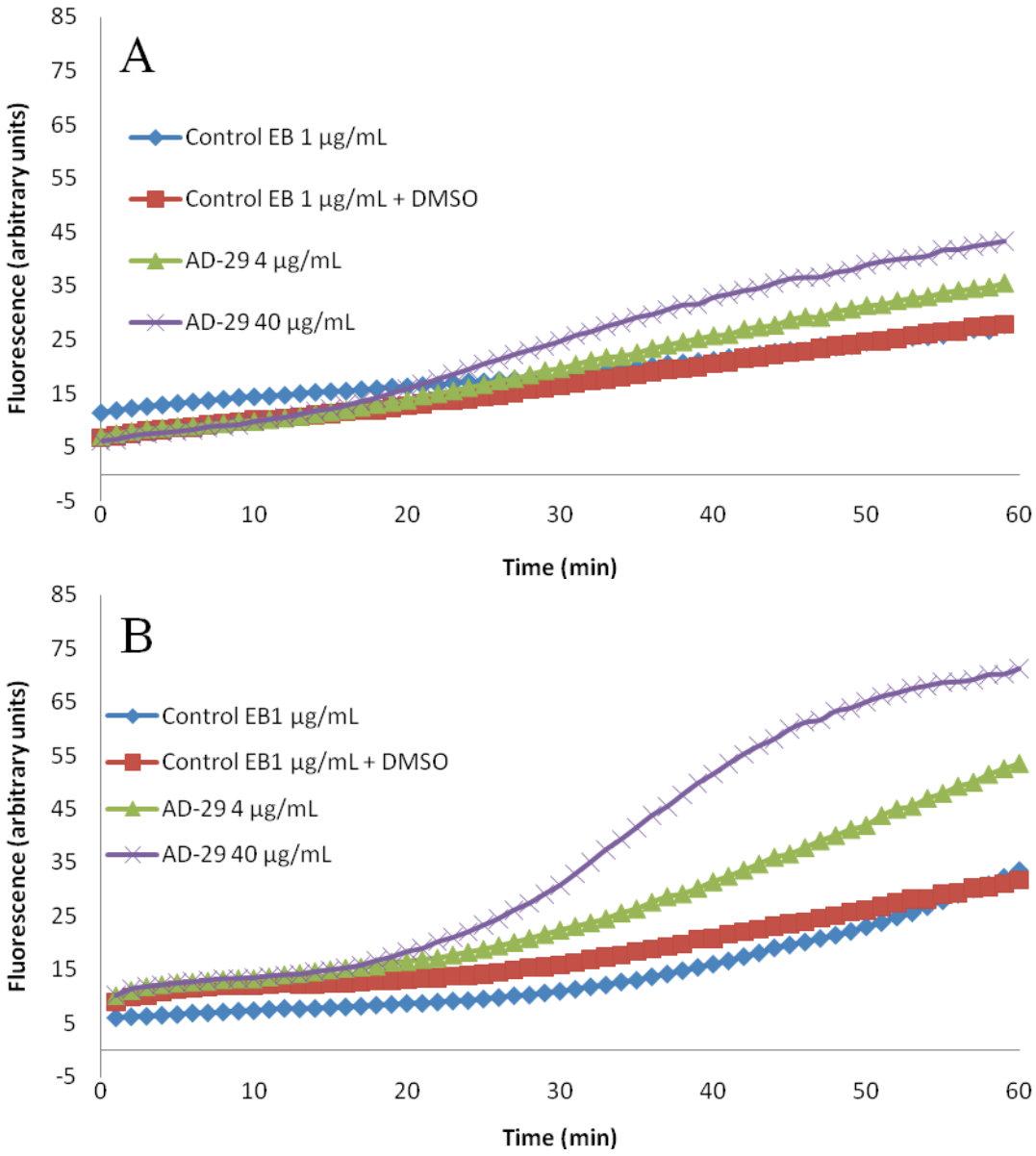

Figure 25. Accumulation of EB (1µg/mL) in the presence of AD-29 (4 and 40 µg/mL) on PAR (A) and MDR (B) cells.

As demonstrated by Figure 25 (A and B), **AD-29** was more effective on MDR cells, however it had a slight effect on PAR cells as well.

The data obtained confirmed the fact that **AD-29** is more selective for the MDR cells which overexpress ABCB1.

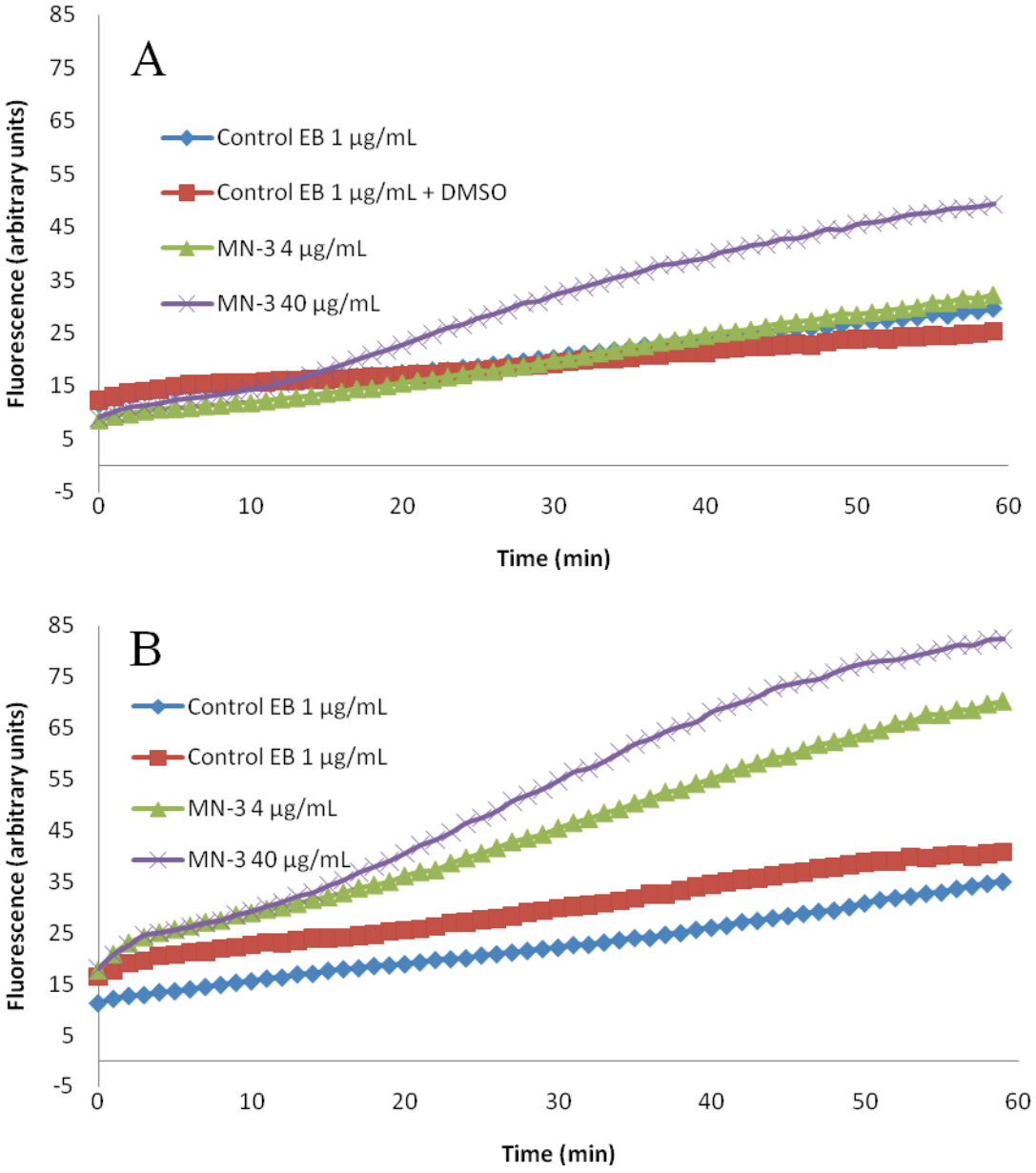

Figure 26. Accumulation of EB (1 µg/mL) in the presence of MN-3 (4 and 40 µg/mL) on PAR (A) and MDR (B) cells.

As demonstrated in Figure 26 (A and B), **MN-3** promoted the accumulation of EB in PAR and MDR cells as well. This compound caused EB accumulation in a dose-dependent manner and was more effective on MDR cells.

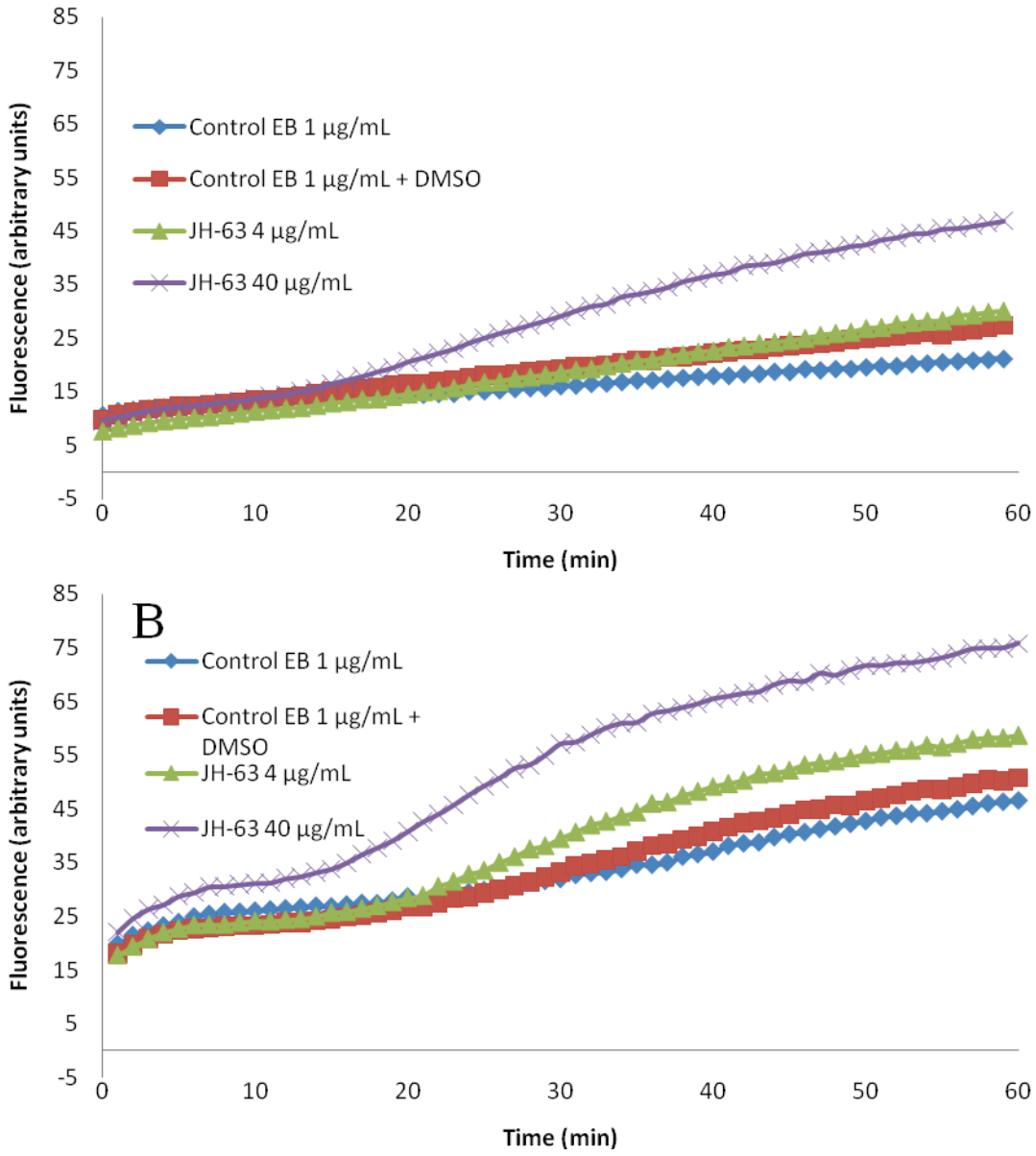

Figure 27. Accumulation of EB (1 µg/mL) in the presence of JH-63 (4 and 40 µg/mL) on PAR (A) and MDR (B) cells.

As shown in Figure 27 (A and B) **JH-63** promoted the retention of EB in MDR and PAR cells as well, dose-dependently.

V – DISCUSSION AND CONCLUSIONS

1.1 - Evaluation of hydantoin derivatives on Gram-negative and Gram-positive bacteria

The increasing prevalence of MDR bacteria is one of the great concerns to health care institutions. This, complemented with the reduced number of new agents entering the clinical practise, is recognised as a major threat to public health (www.dh.gov.uk). Over-expressed efflux pumps that extrude a variety of structurally unrelated antibiotics are associated with multidrug resistant (MDR) phenotypes in Gram-negative bacteria. Usually, the characterization of efflux activity has been essentially restricted to bacteria that overexpress efflux components (SPENGLER *et al,* 2009a). However, the intrinsic efflux pump apparatus of the wild type Gram-negative strain that produces infection provides the means by which the bacterium survives in different physiological conditions (MARTINS *et al,* 2009).

In the present study, thirty hydantoin compounds were evaluated for their efflux modulating effects on the intrinsic efflux system of Gram-negative and Gram-positive bacteria via the use of an automated real-time fluorometric method that monitors the intracellular accumulation of ethidium bromide.

Hydantoins play an important role in purine catabolic pathway that regulates the purine pool in the cell providing precursors for nucleic acid synthesis (AGARWAL *et al,* 2007). In addition, the nucleobase-cation-symport-1 (NCS1) transporters are essential components of salvage pathways for nucleobases and related metabolites, e.g. NCS1 benzyl-hydantoin transporter, Mhp1, from *Microbacterium liquefaciens* (WEYAND *et al,* 2008).

Besides the biochemical processes, hydantoins have pharmacological properties and are used to treat many human diseases (THENMOZHIYAL *et al,* 2004; RAJIC *et al,* 2006). Furthermore, it has been demonstrated that 5-arylidene-2-thiohydantoins have *in vitro* antimycobacterial activity (KIEC-KONONOWICZ & SZYMANSKA, 2002).

In our study, the tested hydantoin derivatives were more effective in the selected strains of Gram-negative bacteria i.e. *Salmonella* Enteritidis NCTC 13349 and *Escherichia coli* AG 100 than in Gram-positives. In spite of their less permeable cell envelope, the tested compounds promoted the accumulation of EB in these bacterial cells. On the contrary, hydantoin derivatives were not effective on the EB accumulation in the selected Gram-positive bacteria.

The best compounds for each bacterial strain are summarized in Table 13. The hydantoin derivatives were selected based on their RFF values. Compounds that presented RFF values above 0.3 were considered the most effective ones.

Table 13. Most effective hydantoin derivatives on the selected bacterial strains.

Bacterial strains	Compounds
S*almonella* Enteritidis NCTC 13349	**Thioam-1**, **SZ-2**, **P3**, **RW-15b**, **AD-26**, **GL-7**, **AD-29**, **GL-18**, **P7** and **SZ-7**
Escherichia coli AG 100	**AD-29**, **RW-15b**, **AD-26**, **Thioam-1**, **GL-18**, **P3**, **KF-2**, **SZ-2** and **RW-13**
Staphylococcus aureus ATCC 25923	**GL-18**, **MN-3** and **GL-16**
Enterococcus faecalis ATCC 29212	**GL-14**, **P3** and **GL-7**

The compounds **Thioam-1**, **SZ-2**, **P3**, **RW-15b**, **AD-26**, **AD-29**, **GL-18**, **GL-7**, **KF-2**, **SZ-7**, **MN-3**, **GL-16** and **GL-14** promoted significantly the accumulation of EB in the selected bacterial strains.

1.2 Future perspectives

The real-time fluorometry is a very useful tool to screen a great number of compounds, in this case a series of hydantoin derivatives, because it provides information about transport kinetics thereby offers a rapid, high-throughput, reproducible, accurate and inexpensive screening of efflux pump inhibitors.

For further studies, different physiological conditions such as different pHs, temperatures and presence/absence of glucose should be investigated in order to mimic the natural environmental factors present in living organisms.

It is well known that a cascade of hydantoinase, *N*-carbamoylase and hydantoinracemase can be used for the production of natural and unnatural chiral D - and L -amino acids from chemically synthesized hydantoin derivatives (ALTENBUCHNER *et al,* 2001).

Concerning our study, we have no information about the metabolism, enzymatic modification or cleavage of the derivatives during the period of the assay. For further studies the role of temperature and pH should be elucidated, as well as the activity of the derivatives in bacteria having over-expressed efflux pump systems.

2.1 Evaluation of hydantoin derivatives on the activity of P-glycoprotein in mouse T-lymphoma cells

Multidrug resistance plays a crucial role in the failure of treatment of cancer. One such mechanism involves the cytoplasmic membrane-localised transport system, which takes part in normal physiological functions as well. In cancer patients, the main reason for treatment failure is the presence of resistance to the chemotherapy. The major mechanism of multidrug resistance is the elevated expression of ATP-dependent drug-efflux pumps, which reduce the accumulation of the anticancer agents. In many tumour cell lines, multidrug resistance is often associated with the over-expression of ABC drug-transporter P-glycoprotein (P-gp), also known as ABCB1. The efflux of chemotherapeutic drugs caused by these transporters, reduce the cellular accumulation of these agents, limiting the success of chemotherapy, applied for the treatment of cancer (AMBUDKAR *et al,* 2005).

Hydantoin derivatives possess a wide range of biochemical effects as well as various pharmacological properties, e.g. anti-tumour activity (KUMAR *et al,* 2009). Although hydantoin compounds have been studied extensively, there are not many studies about their anticancer properties. Recently, the cytotoxic activity of spirohydantoin derivatives was tested in ovarian and breast cancer cells (THENMOZHIYAL *et al,* 2004). It has been shown that a spirohydantoin derivative induces growth inhibition and apoptosis in leukemic cells (KAVITHA *et al,* 2009).

In the present study, the majority of the tested compounds could inhibit the ABCB1 related resistance, causing increased accumulation of EB inside the cells.

It was thus possible to evaluate the inhibition effect of the thirty hydantoin derivatives in L5178 mouse T-cell lymphoma cells (parental, PAR) and its human *ABCB1*-gene transfected sub-line (MDR) overexpressing the ABCB1 transporter. The best compounds, based on their RFF values, are summarized in Table 14. The most effective hydantoin derivatives demonstrated RFF values over 0.3.

Table 14. The most active hydantoin derivatives on parental (PAR) and multidrug resistant (MDR) human MDR1 (ABCB1) gene-transfected mouse lymphoma cells.

Cell line	Most effective compounds
MDR	**PDPH-3**, **GL-7**, **KK-XV**, **AD-29**, **Thioam-1**, **SZ-7**, **KF-2**, **MN-3**, **RW-13**, **LL-9**, **P3**, **AD-26**, **JH-63** and **RW-15b**
PAR	**AD-29**, **KF-2**, **Thioam-1**, **Fur-2**, **Mor-1**, **MN-3**, **P3**, **JH-63**, **SZ-7** and **PDPH-3**

Previously, a semi-automated fluorometric method has been developed by Viveiros *et al.* that utilizes ethidium bromide (EB), a common substrate of bacterial efflux pumps (VIVEIROS *et al,* 2008). The method is sufficiently sensitive to characterize the efflux pump systems of bacteria. Because EB is also recognized and extruded by ATP-binding-cassette (ABC) transporters, the method has been extended for the evaluation of agents that can inhibit the extrusion of EB on a real-

time basis by mouse lymphoma cells containing the human *MDR1(ABCB1)* gene (SPENGLER *et al,* 2009b). Using this method, four curcubitane-type triterpenes isolated from *Momordica balsamina* Linn. (Cucurbitaceae) were evaluated for their activity to inhibit the ABC transporter P-glycoprotein in mouse lymphoma cells. The evaluation was conducted by flow cytometry using rhodamine 123 and real-time fluorometry that assesses accumulation of EB on a real-time basis (SPENGLER *et al,* 2009c).

Based on these studies, a large number of compounds can be screened by real-time fluorometry and selected for further studies.

In the present work, thirty hydantoin compounds were evaluated for their efflux modulating effects in cancer cells using real-time fluorometry based on the intracellular accumulation of ethidium bromide.

It is important to note that many compounds precipitated at higher concentrations (> 100 mg/L) in the media used such as **SZ-2, SZ-7, BS-1, JH-63, MN-3, P7, P10, AD-26, AD-29, RW-13** and **Thioam-1**. Since we applied the compounds at lower concentrations, this fact did not influence the outcomes of the experiments.

The selected compounds were studied by fluorescence activated cell sorting (FACS) using rhodamine 123, a fluorochrome substrate of P-gp (SPENGLER *et al,* 2010).

The mouse lymphoma cell line transfected with the human *MDR1* gene that codes for the ABC transporter P-gp that is responsible for multidrug resistance of this cell line to cytotoxic agents, is a very useful model for evaluating agents that inhibit the activity of the transporter. Among the hydantoin derivatives evaluated for potential inhibition of the P-gp transporter, **BS-1**, **MN-3** and **JH-63** were the most effective inhibitors at the concentration of 4 mg/L.

Surprisingly, on MDR cancer cells the rhodamine 123 retention data did not always correlate with the ethidium bromide accumulation data in terms of the magnitude of activity. In flow cytometry,

compound **BS-1** was the most effective compound but in real-time fluorometry it had a slight effect on the inhibition of ABCB1. On the other hand, **PDPH-3** and **KK-XV** seemed to be promising compounds in the real-time fluorometry, although they had less activity on rhodamine 123 retention in flow cytometry. A possible explanation can be the use of different P-gp subtrates (ethidium bromide and rhodamine 123) in different experimental conditions.

From the most effective compounds, four compounds were chosen to determine their interaction with the anticancer drug doxorubicin. Compounds **SZ-7, BS1, MN-3** and **JH-63** were combined with doxorubicin on *MDR1*-gene transfected mouse lymphoma cells. Compounds **BS-1, MN-3** and **JH-63** showed synergistic effect with doxorubicin on mouse lymphoma cells, furthermore compound **SZ-7** had indifferent effect with doxorubicin (SPENGLER *et al,* 2010).

The most active structures contained aromatic substituents as well as some tertiary amine fragments.

2.2 Future perspectives

Hydantoin derivatives possess a wide range of pharmacological applications and are used to treat many human diseases. One such disease is epilepsy, characterized by the uncontrolled convulsions. The most known hydantoin derivative is phenytoin that is used since 1938 for the treatment of generalized tonic-clonic seizures (THENMOZHIYAL *et al,* 2004).

Various aspects of the biochemical and pharmacological properties of hydantoin derivatives have been studied (THENMOZHIYAL *et al,* 2004; SOMSÁK *et al,* 2001). However, the anticancer activity of these compounds has received little attention.

Based on the results obtained, compounds **AD-26, AD-29, RW-13, KF-2, BS-1, MN-3, RW-15b** and **JH-63,** should be studied for *in vitro* capability of reversing or reducing resistance of the mouse lymphoma *MDR1* gene transfected cell to cytotoxic agents to which they are initially resistant.

To clarify the active group(s) of the hydantoin derivatives and to validate the ABC transporter targets we plan Qualitative Structure Activity Relationship (QSAR) studies in collaboration with French (UMR-MD1, Transporteurs Membranaires, Chimiorésistance et Drug Design, Faculté de Médecine, Marseille) and Polish (Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College, Kraków) research groups.

With the purpose to explore the possibilities of the tested hydantoin derivatives, their activity on different cell lines should be of interest. We plan to investigate the activity of the hydantoin derivatives in other MDR cancer models, such as K562 human leukaemia cells with overexpressed P-gp (ABCB1) or colon carcinoma model using Colo 205/S sensitive colon carcinoma cells with normal ABCB1 expression pattern and Colo 320/R resistant colon carcinoma having overexpressed ABCB1 system.

Active compounds are then to be examined in the mouse model for ability to shrink solid transplanted tumours, and if effective, they would certainly progress to clinical trial status.

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